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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
G01N 33/68, 33/567, C12N 15/16, C07K7/10

A1

(11) International Publication Number: WO 92/15015

(43) International Publication Date: 3 September 1992 (03.09.92)

(21) International Application Number: PCT/US92/01469

(22) International Filing Date: 25 February 1992 (25.02.92)

(30) Priority data:

662,221	25 February 1991 (25.02.91)	US
816,285	31 December 1991 (31.12.91)	US

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(81) Designated States: AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH (European patent), CI (OAPI patent), CM (OAPI patent), CS, DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GA (OAPI patent), GB (European patent), GN (OAPI patent), GR (European patent), HU, IT (European patent), JP, KP, KR, LK, LU (European patent), MC (European patent), MG, ML (OAPI patent), MN, MR (OAPI patent), MW, NL (European patent), NO, PL, RO, RU, SD, SE (European patent), SN (OAPI patent), TD (OAPI patent), TG (OAPI patent).

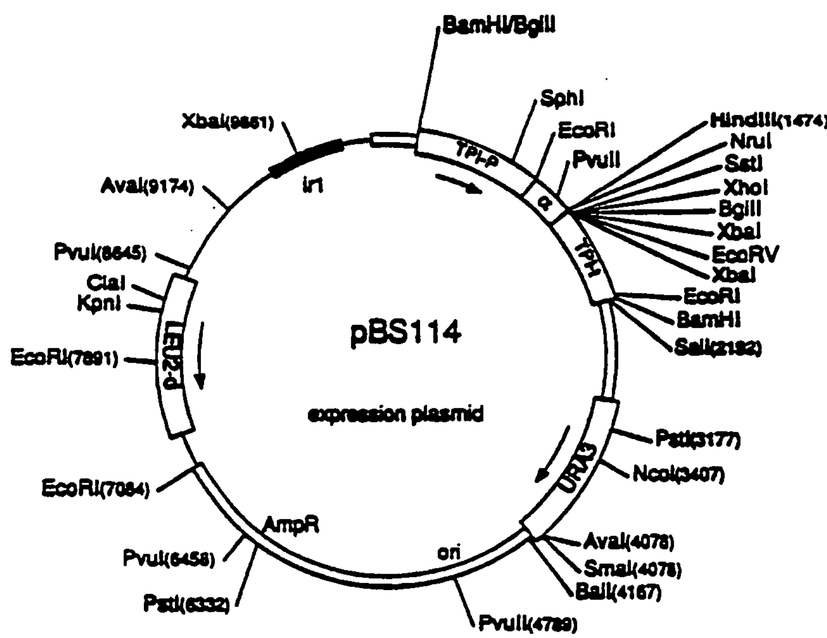
Published

With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: METHODS FOR DETECTING GALANIN ANTAGONISTS

(57) Abstract

DNA molecules encoding human galanin are provided. The molecules are used within methods for detecting galanin antagonists through the use of recombinant DNA techniques. Briefly, DNA sequences encoding galanin analogs are generated and expressed in suitable host cells. The analogs are exposed to a galanin receptor coupled to a response pathway in the presence of native galanin. A reduction in the inhibition of the response pathway resulting from the binding of the galanin analog to the galanin receptor, relative to the inhibition of the response pathway by native galanin alone indicates the presence of a galanin antagonist. Galanin antagonists identified and isolated through these methods are also provided.



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Description

METHODS FOR DETECTING GALANIN ANTAGONISTS

Related Application

The present application is a continuation-in-part of U.S.S.N. 07/662,221, filed February 25, 1991.

Technical Field

The present invention is directed generally toward methods for detecting galanin antagonists, and more specifically, to methods of producing and screening large numbers of potential galanin antagonists through the use of recombinant DNA techniques.

Background of The Invention

Diabetes mellitus, a disease in which a major indicator is an elevated blood glucose level, is generally believed to result from low insulin levels and elevated glucagon levels. However, hyperglycemia in non-insulin dependent diabetes (also known as type II diabetes), in both non-obese and obese patients, has been shown in the presence of both elevated glucagon and insulin levels.

Type II diabetes is a heterogeneous disorder involving pancreatic islet cell dysfunction and insulin resistance. It is characterized by hyperglycemia, a defect in first-phase glucose-induced insulin secretion and impaired glucose utilization. Insulin secretion in response to non-glucose secretagogues is normal or near normal in patients with type II diabetes.

Type II diabetes is treated with sulfonyl ureas, which act on ATP-sensitive K^+ channels, thereby indirectly increasing insulin secretion. These drugs are, however, unsuitable for long term use as patients become increasingly resistant to insulin. It eventually becomes necessary to administer exogenous insulin.

Hyperglycemia may also occur in any state of sympathetic neural activation, including stress due to, for example, surgery, trauma, myocardial infarction or burns. Although insulin is sometimes used therapeutically in such cases, its use creates a significant risk of hypoglycemia.

There remains a need in the art for effective treatments for type II diabetes mellitus and stress-related hyperglycemia. The present invention fulfills this need and provides other related advantages.

Disclosure of Invention

Briefly stated, the present invention provides methods for detecting the presence of galanin antagonists. In one aspect, the methods comprise: (a) growing host cells containing a DNA construct capable of directing the expression of a galanin analog, the construct comprising the following operably linked elements: a transcriptional promoter, a DNA sequence encoding a galanin analog, and a transcriptional terminator, under growth conditions suitable for the expression of the galanin analog; (b) isolating the galanin analog encoded by the DNA sequence from the host cells; (c) exposing the isolated galanin analog in the presence of native galanin to a galanin receptor coupled to a response pathway under conditions and for a time sufficient to allow binding of the galanin analog to the receptor and an associated response through the pathway; and (d) detecting a reduction in the inhibition of the response pathway resulting from the binding of the galanin analog to the galanin receptor, relative to the inhibition of the response pathway by native galanin alone and therefrom determining the presence of a galanin antagonist. In one embodiment, the DNA construct further comprises a secretory signal sequence operably linked to the DNA sequence encoding a galanin analog. Within another embodiment, the galanin receptor is membrane-bound in a

cell-free extract, such as an insulinoma cell membrane preparation. In another embodiment, the galanin receptor is membrane-bound in a whole cell, such as an insulinoma cell.

Within another aspect of the present invention, galanin antagonists are produced from a host cell containing a DNA construct capable of directing the expression of a galanin antagonist, the construct comprising the following operably linked elements: a transcriptional promoter, a DNA sequence encoding a galanin antagonist, wherein the sequence encodes one or more amino acid residues that are different than the corresponding amino acid residues in native galanin, and a transcriptional terminator.

Within a related aspect, the present invention provides isolated DNA molecules encoding human galanin and human galanin antagonists.

These and other aspects will become evident upon reference to the following detailed description and attached drawings.

Brief Description of the Drawings

Figure 1 discloses the sequence of the coding strand of a human galanin cDNA, together with the deduced amino acid sequence (Sequence ID Numbers 1 and 2). The mature peptide extends from glycine, amino acid number 7, through serine, amino acid number 36. Numbers below the lines refer to amino acids; those on the right margin refer to nucleotides.

Figure 2 discloses the representative expression vector pBS114. Abbreviations used include TPI-P, *TPI1* promoter; α, alpha factor signal sequence; TPI-t, *TPI1* terminator; and ir1, inverted repeat 1 of the 2 micron plasmid.

Detailed Description of the Invention

Prior to setting forth the invention, it may be helpful to an understanding thereof to set forth definitions of certain terms to be used hereinafter.

Analog: A molecule, other than a native ligand, capable of being bound by the ligand-binding domain of a receptor. The molecule may be chemically synthesized, produced through recombinant DNA methodology or may occur in nature.

As used herein, galanin analogs are galanin-like polypeptides that contain one or more amino acid residues that are different than the corresponding amino acid residues in native galanin and that are capable of binding to a galanin receptor. These differences may comprise deletions, additions and/or substitutions of amino acids relative to native galanin.

Response pathway: A response pathway is a biochemical pathway activated in response to external stimuli that is generally, but not always, directly coupled to a membrane-bound receptor. Response pathways generally regulate cellular functions such as extracellular matrix secretion, hormone secretion, chemotaxis, differentiation, and cell division in responsive cells. One such response pathway is the adenylate cyclase response pathway, which is coupled to the membrane-bound galanin receptor. The adenylate cyclase response pathway is inhibited upon binding of galanin to its cellular receptor, thereby reducing intracellular concentrations of cyclic AMP (cAMP).

Antagonist: A molecule capable of binding to a receptor, but that does not affect or exhibits a reduced effect on a response pathway within a cell as compared to the native ligand. Galanin antagonists are generally identified by their ability to bind to the galanin receptor and their inability or reduced ability to affect a cellular response pathway. For example, putative galanin antagonists are combined with native galanin and the inhibition of forskolin-stimulated cAMP production or

insulin secretion is measured. Galanin antagonists are identified as those molecules that reduce the inhibition of cAMP production or insulin secretion relative to native galanin alone.

<u>DNA Construct</u>: A DNA molecule, or a clone of such a molecule, either single- or double-stranded that has been modified through human intervention to contain segments of DNA combined and juxtaposed in a manner that as a whole would not otherwise exist in nature.

DNA constructs may contain the information necessary to direct the expression of DNA sequences encoding polypeptides of interest. Such DNA constructs, known as expression vectors, will generally include transcriptional promoters, enhancers and transcriptional terminators. DNA constructs containing the information necessary to direct the secretion of a polypeptide will also contain at least one secretory signal sequence.

<u>Secretory Signal Sequence</u>: A DNA sequence encoding a secretory peptide. A secretory peptide is an amino acid sequence that acts to direct the secretion of a mature polypeptide or protein from a cell. Secretory peptides are generally characterized by a core of hydrophobic amino acids and are typically (but not exclusively) found at the amino termini of newly synthesized proteins. Very often the secretory peptide is cleaved from the mature protein during secretion. Such secretory peptides contain processing sites that allow cleavage of the secretory peptides from mature proteins as they pass through the secretory pathway. Processing sites may be encoded within the secretory peptide or may be added to the secretory peptide by, for example, <u>in vitro</u> mutagenesis. Certain secretory peptides may be used in concert to direct the secretion of polypeptides and proteins. One such secretory peptide that may be used in combination with other secretory peptides is the third domain of the yeast Barrier protein (disclosed in U.S.

patent application Serial No. 07/270,933, which is incorporated herein by reference).

The present invention provides isolated DNA molecules encoding human galanin, a 30-amino acid sympathetic neurotransmitter that is believed to mediate hormone secretion by pancreatic islet cells. Experimental evidence indicates that galanin opens ATP-sensitive K^+ channels, inhibits adenylate cyclase and directly interferes with exocytosis. These effects may individually and collectively inhibit insulin secretion. Recent studies by Dunning et al. (Diabetes 39 (Suppl.): 135A, 1990) suggest that galanin is indeed a regulator of insulin secretion in humans. These DNA molecules are therefore useful in generating galanin antagonists that may be used to release pancreatic islet cells from hormonal inhibition of insulin secretion, thereby providing a more direct treatment for type II diabetes.

As noted above, an object of the present invention is to provide methods for detecting galanin antagonists using recombinant methods and host cells. The present invention provides the ability to produce galanin analogs from transformed or transfected host cells. The analogs are exposed, in the presence of native galanin, to a galanin receptor coupled to a response pathway. A reduction in the inhibition of the response pathway as compared to the inhibition obtained using native galanin alone is indicative of the presence of a galanin antagonist. The reduced inhibition of a response pathway is seen as, for example, increased secretion of insulin or somatostatin, or increased cAMP production as compared to galanin-treated cells. Galanin analogs produced according to the present invention may be screened in high through-put antagonist screens. By using recombinant DNA methods, the present invention also provides a method for screening pools of galanin analogs within such high through-put screens to identify galanin antagonists. The present

invention also provides methods for directly producing galanin antagonists through the use of recombinant host cells.

Also as noted above, the present invention provides methods for producing large numbers of galanin analogs through the use of pools of DNA sequences encoding such analogs. Galanin coding sequences may be produced synthetically using standard techniques or may be cloned from, for example, pheochromocytoma cells (e.g. Bauer et al., J. Clin. Endocrinol. Metab. 63: 1372-1378, 1986), using standard cloning methods such as those described by Maniatis et al. (Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, NY, 1982; which is incorporated herein by reference), Sambrook et al. (Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, NY, 1989; which is incorporated herein by reference) or Mullis et al. (U.S. Patent No. 4,683,195; incorporated herein by reference). As disclosed in more detail hereinafter, a galanin cDNA was cloned from human pheochromocytoma tissue using the polymerase chain reaction method of Mullis et al. (ibid.) using primers based on sequence conservation among published nucleotide and amino acid sequences for bovine, porcine and rat galanins. Poly $(A)^+$ RNA was prepared from pooled pheochromocytoma specimens. The RNA template was annealed to a synthetic oligonucleotide primer, first strand cDNA was synthesized, and galanin sequences were amplified. A clone encoding 27 amino acids of mature galanin and part of the C-terminal propeptide was identified. Such partial clones are extended according to conventional methods (e.g., the RACE procedure of Frohman et al., Proc. Natl. Acad. Sci. USA 85: 8998-9002, 1988, which is incorporated herein by reference) to obtain full-length clones. A genomic galanin clone may also be obtained by polymerase chain reaction amplification.

Pools of DNA sequences encoding galanin analogs may be generated by saturation mutagenesis of a DNA

sequence encoding galanin (using, for example, the methods described by Little [Gene 88:113-115, 1990] or Hermes et al. [Gene 88:143-151, 1989]), or segment-directed mutagenesis (as described, for example, by Shortle et al., [Proc. Natl. Acad. Sci. USA 77:5375-5379, 1980]). Alternatively, pools of galanin analogs may be generated by forced nucleotide misincorporation as described by, for example, Liao and Wise (Gene 88:107-111, 1990). Briefly, Liao and Wise describe a method for introducing random point mutations into cloned DNA fragments via the forced misincorporation of deoxynucleoside triphosphates by either a reverse transcriptase or a mutant T7 DNA polymerase. In combination with specific primers and limiting amounts of non-mutagenic nucleoside triphosphates, these two polymerases, which lack proofreading activity, incorporate incorrect nucleotides into the primed sequence and provide a wide spectrum of random mutations in a given sequence.

In a preferred embodiment, pools of DNA sequences encoding galanin analogs are generated by synthesizing randomly mutagenized oligonucleotides using, for example, the method described by Hutchinson et al. (Proc. Natl. Acad. Sci. USA 83:710-714, 1986). To facilitate subsequent cloning, it is preferred that oligonucleotides encoding galanin analogs be synthesized to form adapters upon hybridization such that the galanin analog coding sequences are flanked by adhesive ends. It may be particularly preferred to add a sequence encoding a bridging region which allows the in-frame fusion of sequences encoding a secretory signal sequence and a galanin coding sequence. Galanin analog coding sequences are preferably synthesized on an oligonucleotide synthesizer by cross contaminating the reagent bottles that normally contain pure phosphoramidites corresponding to the bases A, G, C, and T at low levels with each of the other bases. Cross contamination of the reagent bottles may be achieved by adding between .01% and 14% of each

incorrect base, with a cross contamination of between 0.8% and 2% being preferred, and 1% being particularly preferred. A 1% cross contamination with each incorrect base will theoretically lead to approximately 2.5 base changes per molecule.

The galanin analog coding sequences are inserted into a suitable expression vector to produce a library that is in turn introduced by transfection or transformation into a host cell. Expression vectors for use in carrying out the present invention will comprise a promoter capable of directing the transcription of a cloned DNA and a transcriptional terminator. Host cells for use in practicing the present invention include mammalian, avian, plant, insect, bacterial and fungal cells. Fungal cells, including species of yeast (e.g., Saccharomyces spp., Schizosaccharomyces spp., Kluyveromyces spp.) or filamentous fungi (e.g., Aspergillus spp., Neurospora spp.) may be used as host cells within the present invention. Strains of the yeast Saccharomyces cerevisiae are particularly preferred.

Within the present invention it is preferred to express galanin analogs in eukaryotic host cells that can secrete the analogs into the culture media. To direct the galanin analogs into the secretory pathway of the host cell at least one secretory signal sequence is operably linked to the galanin analog DNA sequence. Preferred secretory signals include the galanin secretory signal (pre-pro sequence), the alpha factor signal sequence (pre-pro sequence; Kurjan and Herskowitz, Cell 30:933-943, 1982; Kurjan et al., U.S. Patent No. 4,546,082; Brake, EP 116,201), the PHO5 signal sequence (Beck et al., WO 86/00637), the BAR1 secretory signal sequence (MacKay et al., U.S. Patent No. 4,613,572; MacKay, WO 87/002670), the SUC2 signal sequence (Carlson et al., Mol. Cell. Biol. 3:439-447, 1983), the α-1-antitrypsin signal sequence (Kurachi et al., Proc. Natl. Acad. Sci. USA 78:6826-6830, 1981), the α-2 plasmin inhibitor signal sequence (Tone et

al., J. Biochem. (Tokyo) 102:1033-1042, 1987) and the tissue plasminogen activator signal sequence (Pennica et al., Nature 301:214-221, 1983). Alternatively, a secretory signal sequence may be synthesized according to the rules established, for example, by von Heinje (Eur. J. Biochem. 133:17-21, 1983; J. Mol. Biol. 184:99-105, 1985; Nuc. Acids Res. 14:4683-4690, 1986).

Secretory signal sequences may be used singly or may be combined. For example, a first secretory signal sequence may be used singly or in combination with a sequence encoding the third domain of the Saccharomyces cerevisiae Barrier protein (described in U.S. Patent Application Serial No. 07/270,933, which is incorporated by reference herein in its entirety). The third domain of Barrier may be positioned in proper reading frame 3′ of the galanin analog DNA sequence or 5′ to the DNA sequence and in proper reading frame with both the secretory signal sequence and the galanin analog DNA sequence.

Suitable yeast vectors for use in the present invention include YRp7 (Struhl et al., Proc. Natl. Acad. Sci. USA 76:1035-1039, 1978), YEp13 (Broach et al., Gene 8:121-133, 1979), POT vectors (Kawasaki et al, U.S. Patent No. 4,931,373, which is incorporated by reference herein), pJDB249 and pJDB219 (Beggs, Nature 275:104-108, 1978) and derivatives thereof. Such vectors will generally include a selectable marker, which may be one of any number of genes that exhibit a dominant phenotype for which a phenotypic assay exists to enable transformants to be selected. Preferred selectable markers are those that complement host cell auxotrophy, provide antibiotic resistance or enable a cell to utilize specific carbon sources, and include LEU2 (Broach et al., ibid.), URA3 (Botstein et al., Gene 8:17, 1979), HIS3 (Struhl et al., ibid.) or POT1 (Kawasaki et al., ibid.). Another suitable selectable marker is the CAT gene, which confers chloramphenicol resistance on yeast cells.

Preferred promoters for use in yeast include promoters from yeast glycolytic genes (Hitzeman et al., J. Biol. Chem. 255:12073-12080, 1980; Alber and Kawasaki, J. Mol. Appl. Genet. 1:419-434, 1982; Kawasaki, U.S. Patent No. 4,599,311) or alcohol dehydrogenase genes (Young et al., in Genetic Engineering of Microorganisms for Chemicals, Hollaender et al., (eds.), p. 355, Plenum, New York, 1982; Ammerer, Meth. Enzymol. 101:192-201, 1983). In this regard, particularly preferred promoters are the TPI1 promoter (Kawasaki, U.S. Patent No. 4,599,311, 1986) and the ADH2-4^{c} promoter (Russell et al., Nature 304:652-654, 1983; Irani and Kilgore, European Patent Office Publication EP 284,044, which is incorporated herein by reference). The expression units may also include a transcriptional terminator. A preferred transcriptional terminator is the TPI1 terminator (Alber and Kawasaki, ibid.).

In addition to yeast, galanin analogs can be expressed in filamentous fungi, for example, strains of the fungi Aspergillus (McKnight et al., U.S. Patent No. 4,935,349, which is incorporated herein by reference). Examples of useful promoters include those derived from Aspergillus nidulans glycolytic genes, such as the ADH3 promoter (McKnight et al., EMBO J. 4:2093-2099, 1985) and the tpiA promoter. An example of a suitable terminator is the ADH3 terminator. The expression units utilizing such components are cloned into vectors that are capable of insertion into the chromosomal DNA of Aspergillus.

Techniques for transforming fungi are well known in the literature, and have been described, for instance, by Beggs (ibid.), Hinnen et al. (Proc. Natl. Acad. Sci. USA 75:1929-1933, 1978), Yelton et al. (Proc. Natl. Acad. Sci. USA 81:1740-1747, 1984), and Russell (Nature 301:167-169, 1983). The genotype of the host cell will generally contain a genetic defect that is complemented by the selectable marker present on the expression vector. Choice of a particular host and selectable marker is well

within the level of ordinary skill in the art. To optimize production of heterologous proteins, it is preferred that the host strain carry a mutation, such as the yeast pep4 mutation (Jones, Genetics 85:23-33, 1977), which results in reduced proteolytic activity.

In addition to fungal cells, cultured mammalian cells may be used as host cells within the present invention. Preferred cultured mammalian cells for use in the present invention include the COS-1 (ATCC CRL 1650), BHK, and 293 (ATCC CRL 1573; Graham et al., J. Gen. Virol. 36:59-72, 1977) cell lines. A preferred BHK cell line is the BHK 570 cell line (deposited with the American Type Culture Collection under accession number CRL 10314). In addition, a number of other mammalian cell lines may be used within the present invention, including Rat Hep I (ATCC CRL 1600), Rat Hep II (ATCC CRL 1548), TCMK (ATCC CCL 139), Human lung (ATCC CCL 75.1), Human hepatoma (ATCC HTB-52), Hep G2 (ATCC HB 8065), Mouse liver (ATCC CCL 29.1), NCTC 1469 (ATCC CCL 9.1) and DUKX cells (Urlaub and Chasin, Proc. Natl. Acad. Sci USA 77: 4216-4220, 1980).

Mammalian expression vectors for use in carrying out the present invention will include a promoter capable of directing the transcription of a cloned gene or cDNA. Preferred promoters include viral promoters and cellular promoters. Viral promoters include the immediate early cytomegalovirus promoter (Boshart et al., Cell 41:521-530, 1985) and the SV40 promoter (Subramani et al., Mol. Cell. Biol. 1:854-864, 1981). Cellular promoters include the mouse metallothionein-1 promoter (Palmiter et al., U.S. Patent No. 4,579,821), a mouse V_K promoter (Bergman et al., Proc. Natl. Acad. Sci. USA 81:7041-7045, 1983; Grant et al., Nuc. Acids Res. 15:5496, 1987) and a mouse V_H promoter (Loh et al., Cell 33:85-93, 1983). A particularly preferred promoter is the major late promoter from Adenovirus 2 (Kaufman and Sharp, Mol. Cell. Biol. 2:1304-13199, 1982). Such expression vectors may also contain a set of RNA splice sites located downstream from

the promoter and upstream from the DNA sequence to be expressed. Preferred RNA splice sites may be obtained from adenovirus and/or immunoglobulin genes. Also contained in the expression vectors is a polyadenylation signal located downstream of the coding sequence of interest. Suitable polyadenylation signals include the early or late polyadenylation signals from SV40 (Kaufman and Sharp, ibid.), the polyadenylation signal from the adenovirus 5 E1B region and the human growth hormone gene terminator (DeNoto et al., Nuc. Acids Res. 9:3719-3730, 1981). The expression vectors may include a noncoding viral leader sequence, such as the adenovirus 2 tripartite leader, located between the promoter and the RNA splice sites. Preferred vectors may also include enhancer sequences, such as the SV40 enhancer and the mouse μ enhancer (Gillies, Cell 33:717-728, 1983). Expression vectors may also include sequences encoding the adenovirus VA RNAs.

Cloned DNA sequences may be introduced into cultured mammalian cells by, for example, calcium phosphate-mediated transfection (Wigler et al., Cell 14:725, 1978; Corsaro and Pearson, Somatic Cell Genetics 7:603, 1981; Graham and Van der Eb, Virology 52:456, 1973.) Other techniques for introducing cloned DNA sequences into mammalian cells, such as electroporation (Neumann et al., EMBO J. 1:841-845, 1982), may also be used. In order to identify cells that have integrated the cloned DNA, a selectable marker is generally introduced into the cells along with the gene or cDNA of interest. Preferred selectable markers for use in cultured mammalian cells include genes that confer resistance to drugs, such as neomycin, hygromycin, and methotrexate. The selectable marker may be an amplifiable selectable marker. A preferred amplifiable selectable marker is the DHFR gene. Selectable markers are reviewed by Thilly (Mammalian Cell Technology, Butterworth Publishers, Stoneham, MA, which is incorporated herein by reference). The choice of

selectable markers is well within the level of ordinary skill in the art.

Selectable markers may be introduced into the cell on a separate plasmid at the same time as the gene of interest, or they may be introduced on the same plasmid. If on the same plasmid, the selectable marker and the gene of interest may be under the control of different promoters or the same promoter, the latter arrangement producing a dicistronic message. Constructs of this type are known in the art (for example, Levinson and Simonsen, U.S. Patent No. 4,713,339). It may also be advantageous to add additional DNA, known as "carrier DNA" to the mixture which is introduced into the cells.

Transfected mammalian cells are allowed to grow for a period of time, typically 1-2 days, to begin expressing the DNA sequence(s) of interest. Drug selection is then applied to select for growth of cells that are expressing the selectable marker in a stable fashion. For cells that have been transfected with an amplifiable selectable marker, the drug concentration may be increased in a stepwise manner to select for increased copy number of the cloned sequences, thereby increasing expression levels.

Preferred prokaryotic host cells for use in carrying out the present invention are strains of the bacteria <u>Escherichia coli</u>, although <u>Bacillus</u> and other genera are also useful. Techniques for transforming these hosts and expressing foreign DNA sequences cloned therein are well known in the art (see, e.g., Maniatis et al., <u>Molecular Cloning: A Laboratory Manual</u>, Cold Spring Harbor Laboratory, 1982; which is incorporated herein by reference, or Sambrook et al., <u>Molecular Cloning: A Laboratory Manual, Second Edition</u>, Cold Spring Harbor, NY, 1989; which is incorporated herein by reference). Vectors used for expressing cloned DNA sequences in bacterial hosts will generally contain a selectable marker, such as a gene for antibiotic resistance, and a promoter that

functions in the host cell. Appropriate promoters include the trp (Nichols and Yanofsky, Meth. Enzymol. 101:155-164, 1983), lac (Casadaban et al., J. Bacteriol. 143:971-980, 1980), and phage λ (Queen, J. Mol. Appl. Genet. 2:1-10, 1983) promoter systems. Plasmids useful for transforming bacteria include pBR322 (Bolivar et al., Gene 2:95-113, 1977), the pUC plasmids (Messing, Meth. Enzymol. 101:20-78, 1983; Vieira and Messing, Gene 19:259-268, 1982), pCQV2 (Queen, ibid.), and derivatives thereof. Plasmids may contain both viral and bacterial elements.

Given the teachings provided herein, promoters, terminators and methods for introducing expression vectors encoding galanin analogs of the present invention into plant, avian and insect cells would be evident to those of skill in the art. The use of baculoviruses, for example, as vectors for expressing heterologous DNA sequences in insect cells has been reviewed by Atkinson et al. (Pestic. Sci. 28:215-224,1990). The use of Agrobacterium rhizogenes as vectors for expressing genes in plant cells has been reviewed by Sinkar et al. (J. Biosci.(Bangalore) 11:47-58, 1987).

Individual transformants or transfectants expressing the galanin analogs are then cloned. In the case of Saccharomyces cerevisiae transformants, individual transformants may be picked onto selective media using sterile toothpicks. In the case of cultured mammalian cell transfectants, individual transfectants may be isolated by cylinder cloning into multi-well culture plates.

The cloned cells are then used in assays that will generally include the steps of (a) growing host cells containing a DNA construct capable of directing the expression of a galanin analog, the construct comprising the following operably linked elements: a transcriptional promoter, a DNA sequence encoding a galanin analog, and a transcriptional terminator, under growth conditions suitable for the expression of the galanin analog;

(b) isolating the galanin analog encoded by the DNA sequence from the host cells; (c) exposing the isolated galanin analogs in the presence of native galanin to a galanin receptor coupled to a response pathway under conditions and for a time sufficient to allow binding of the galanin analog to the receptor and an associated response through the pathway; and (d) detecting a reduction in the inhibition of the response pathway resulting from the binding of the galanin analog to the galanin receptor relative to the inhibition of the response pathway by native galanin, and therefrom determining the presence of a galanin antagonist.

The transfected or transformed cells are grown according to standard methods in a growth medium containing nutrients required for growth of the particular host cells. A variety of suitable media are known in the art and generally include a carbon source, a nitrogen source, essential amino acids, vitamins, minerals and growth factors. The growth medium will generally select for cells containing the DNA construct by, for example, drug selection or deficiency in an essential nutrient which is complemented by the selectable marker on the DNA construct or co-transfected with the DNA construct.

Suitable growth conditions for yeast cells, for example, include culturing in a medium comprising a nitrogen source (e.g. yeast extract or nitrogen-containing salts), inorganic salts, vitamins and essential amino acid supplements as necessary at a temperature between 4°C and 37°C, with 30°C being particularly preferred. The pH of the medium is preferably maintained at a pH greater than 2 and less than 8, more preferably pH 5-6. Methods for maintaining a stable pH include buffering and constant pH control, preferably through the addition of sodium hydroxide. Preferred buffering agents include succinic acid and Bis-Tris (Sigma Chemical Co., St. Louis, MO). Cultured mammalian cells are generally cultured in commercially available serum-containing or serum-free

media. Selection of a medium appropriate for the particular cell line used and determination of suitable growth condition are within the level of ordinary skill in the art.

Galanin analogs expressed by the host cells are then isolated from the cells. If the analogs are secreted by the cells, it will generally be sufficient to remove the cells from the culture media (e.g. by centrifugation or filtration) and assay media samples as described herein. If the galanin analogs are retained within the host cells it will be necessary to lyse the cells and recover the analog(s) from the lysate.

Conditions and times sufficient for the binding of the galanin analog to the receptor will vary with the source of the receptor and the particular assay used; however, conditions suitable for binding will generally be between 4°C and 55°C, preferably 30°C-40°C, under physiological conditions. As used herein, "physiological conditions" indicates conditions approximating the normal environment of a cell-associated galanin receptor, and includes cell culture media and buffered, low-salt solutions within a pH range of between 5 and 9, preferably between 6.8 and 8. Sufficient time for the binding and response will be between 5 and 60 minutes after exposure, with 15-30 minutes being particularly preferred.

As noted above, antagonists are capable of binding to a cellular receptor of the corresponding native ligand, but either are incapable of affecting a response pathway or exhibit a reduced effect on a response pathway as compared to the native ligand. In one embodiment, galanin antagonists are identified through their ability to bind to a cellular galanin receptor and their inability or reduced ability to inhibit the adenylate cyclase response pathway that is stimulated by forskolin or gastric inhibitory polypeptide (GIP). In another embodiment, galanin antagonists are identified through their ability to reduce galanin inhibition of insulin

release or somatostatin release. Galanin receptors have been reported in a number of cells and tissues, for example pancreatic β-cells, insulinoma cells, gut cells, pituitary tissue and brain tissue. Adenylate cyclase activity assays or insulin secretion assays may be carried out using, for example, the method described by Amiranoff et al. (Eur. J. Biochem. 177: 147-152, 1988; incorporated herein by reference). Briefly, RINm5 cells, preferably RIN5mAH T_2 B (RIN5), cells are incubated in culture media in the presence of 0.1 mM forskolin or 0.1 μm GIP for 30 minutes at 37°C to stimulate cAMP production. cAMP production in the control cells is compared to that in cells cultured in the presence of forskolin or GIP plus galanin or galanin and a galanin analog. Cyclic AMP is extracted by adding 1 M perchloric acid, succinylated, and assayed by radioimmunoassay. It is generally preferred that cAMP production be measured using a commercially available kit (e.g. the Scintillation Proximity Assay manufactured by Amersham Corp., Arlington Heights, IL). Using such a kit, the production of cAMP is determined by competition of iodinated-cAMP with anti-cAMP antibodies. Insulin secretion is assayed in forskolin- or GIP-stimulated RIN5 cells by radioimmunoassay as disclosed by Amiranoff et al. (ibid.) or Vallar et al. (J. Biol. Chem. 262: 5049-5056, 1987) or may be assayed using a commercially available kit (e.g. the Scintillation Proximity Assay manufactured by Amersham Corp., Arlington Heights, IL). Briefly, target cells are incubated in medium containing an insulin secretagogue (e.g. forskolin or GIP) with or without galanin and galanin antagonists for 30-60 minutes at 37°C. The medium is then collected, and insulin secretion is quantitated. An insulin RIA kit is available from Novo Nordisk (Bagsvaerd, Denmark) or insuling may be assayed using the Scintillation Proximity Assay (Amersham Corp.). Somatostatin release is assayed as disclosed by Amiranoff et al. (Eur. J. Pharmacol. 191: 401-405, 1990) by measuring the inhibition of basal

somatostatin release by RIN5 cells incubated in the presence of galanin (10^{-10}-10^{-7}M). Assays may also be carried out on other cell types, such as hamster pancreatic β-cell tumor cells (Amiranoff et al., Eur. J. Biochem. 159: 353, 1986; Amiranoff et al., Endocrinology 121: 284-289, 1987) or other cells having galanin receptors (e.g. cultured human pancreatic islet cells) or on isolated β-cell membranes.

In the alternative, a rat galanin analog library is constructed and tested as generally described above. The rat galanin sequence is disclosed by Kaplan et al. (Proc. Natl. Acad. Sci. USA 85: 1065-1069, 1988) and Vrontakis et al. (J. Biol. Chem. 262: 16755-16758), which are incorporated herein by reference. Amino acid changes found to produce rat galanin antagonists are then used to design corresponding human analogs, which are tested for antagonist activity.

The activity of human galanin analogs showing antagonist activity on cultured cells or isolated cell membranes may be confirmed by in vivo testing of substantially pure galanin antagonists, such as by systemic administration or using a whole animal infused pancreas model or in an isolated pancreas model. Substantially pure human galanin analogs are those that are of at least about 50% purity, at least about 70-80% more preferred, and 95-99% or more homogeneity most preferred. Methods for assaying galanin in a whole animal infused pancreas model are described in more detail herein and, in addition, are disclosed by Dunning et al. (Am. J. Physiol. 251 (Endocrinol. Metab. 14): E127-133, 1986), Dunning and Taborsky (Diabetologia 33: 125-126, 1990), Dunning et al. (Am. J. Physiol. 258 (Endocrinol. Metab. 21): E436-E444, 1990), and Dunning et al. (Am. J. Physiol. 256 (Endocrinol. Metab. 19): E191-E198, 1989), which are incorporated herein by reference. Isolated pancreas models are disclosed by Kwok et al. (Gastroenterology 88:90-95, 1988), Penhos et al., Diabetes 18: 733-738,

1969) and Takemura et al., Am. J. Med. 8 (Suppl 6B): 65, 1986), which are incorporated herein by reference.

Once a cloned cell line or strain expressing a galanin antagonist is identified, the expression vector may be isolated and the insert (antagonist coding sequence) sequenced to confirm the presence of a mutation. Antagonist coding sequences may be tranferred to other expression vectors as desired and strains or cell lines expressing the antagonist are scaled up for production. Sequence information from different galanin antagonists facilitates the design and construction of additional sequences encoding antagonists with multiple amino acid differences.

The galanin antagonists of the present invention may be purified by ion-exchange and partition chromatography as described by, for example, Coy et al. (Peptides Structure and Function, Pierce Chemical Company, Rockford, IL, pp. 369-372, 1983), by high performance liquid chromatography as described, for example, by Lagny-Pourmir et al. (Peptides 10: 757-761, 1989) or Fisone et al. (Proc. Natl. Acad. Sci. USA 86: 9588-9591, 1989). Additional purification may be achieved by conventional chemical purification means, such as liquid chromatography, gradient centrifugation, and gel electrophoresis, among others. Methods of protein purification are known in the art (see generally, Scopes, R., Protein Purification, Springer-Verlag, NY (1982), which is incorporated herein by reference) and may be applied to the purification of the recombinant galanin analogs described herein.

Alternatively, galanin antagonists identified using the screening methods described herein may be synthesized following any suitable method, such as by exclusively solid-phase techniques, by partial solid-phase techniques, by fragment condensation or by classical solution addition. Synthetic galanin antagonsists of the present invention may be prepared by hand synthesis or

using a suitable peptide synthesizer. In a preferred embodiment of the invention, the polypeptides are synthesized on an Applied Biosystems Model 431A peptide synthesizer.

Solid phase peptide synthesis is the preferred method for preparing polypeptides of the present invention. A particularly preferred method for peptide synthesis is by the Fmoc method essentially described by Carpino and Han (J. Amer. Chem. Soc. 92: 5748-5749, 1970; J. Org. Chem. 37: 3404-3409, 1972). Briefly, solid phase synthesis proceeds from the C-terminus of the peptide to the N-terminus and begins with the coupling of an activated, Fmoc-protected, α-amino acid to a suitable resin. Suitable resins have been described, for example, by Wang et al. (J. Am. Chem. 95: 1328-1333, 1973), Rink (Tetrahedron Lett. 28: 3787-3790, 1987), Breipohl et al. (Tetrahedron Lett. 28: 5651-5654, 1987), Funakoshi et al., J. Am. Soc. Chem. Commun. 11: 382-384, 1988), and Mergler et al. (Peptides, Chemistry and Biology, Proceedings of the Tenth American Peptide Symposium, Marshall, ed., ESCOM, Leiden, pp 259-2660, 1988). A preferred resin is HMP-resin (4-hydroxymethylphenoxymethylcopolystyrene-1% divinylbenzene resin) developed by Wang et al. (ibid.). Fmoc-protected α-amino acids are activated using any of a variety of suitable activation methods known in the art, such as conventional N,N'-dicyclohexylcarbodiimide (DCC) activation, symmetric anhydride activation and 1-hydroxybenzotriazole (HOBt)/DCC activation. A particularly preferred method of activation of the first amino acid of the polypeptide is the symmetric anhydride method. Subsequent α-amino acids are preferably activated using the HOBt/DCC activation method essentially described by Konig and Geiger (Chem. Ber. 103: 788-798, 1970).

Subsequent to the coupling of the Fmoc-protected, C-terminal α-amino acid, the Fmoc protecting group is removed by treatment with 20% piperidine followed by a wash with N-methylpyrrolidone (NMP). Subsequent

activated α-amino acids, preferably as HOBt active esters, are added in the desired order in cycles consisting of activated α-amino acid addition followed by piperidine deprotection. An alternative to adding amino acids to the 'polypeptide in sequential order, is the addition of amino acids that have been coupled prior to addition to the growing polypeptide.

The polypeptide may be removed from the resin, following deprotection of the terminal amino acid, by cleavage with, for example, trifluoroacetic acid. The peptide may be purified by reverse-phase high-pressure liquid chromatography.

It will be understood by those skilled in the art that galanin analogs may be chemically synthesized, such as by the solid-phase method of Barany and Merrifield (in The Peptides Vol. 2A, Gross and Meienhofer, eds, Academic Press, NY, pp. 1-284, 1979) or by use of an automated peptide synthesizer. Although it is possible to synthesize a library of galanin analogs based on the sequence of the native polypeptide disclosed herein and to test those analogs as generally described above, it is more practical to utilize a recombinant polypeptide screening system to identify antagonists.

Substantially pure galanin antagonists of at least about 50% purity are preferred, at least about 70-80% more preferred, and 95-99% or more homogeneity most preferred, particularly for pharmaceutical uses. Once purified, partially or to homogeneity as desired, the recombinant galanin analogs may be used therapeutically. In general, the antagonists of the present invention are administered by injection (intravenous or subcutaneous), inhalation or infusion to patients suffering from inter alia, type II diabetes or stress-related hyperglycemia. The antagonists of the present invention may be present as free bases or as acid salts. Suitable salts will be pharmaceutically acceptable and include metal salts, including alkali and alkaline earth metal salts such as

potassium or sodium salts. Other pharmaceutically acceptable salts include salts of citric, succinic, lactic, hydrochloric and hydrobromic acids. Parenteral compositions may be formulated in aqueous isotonic solutions of between pH 5.6 and 7.4. Suitable isotonic solutions include sodium chloride, dextrose, boric acid, sodium tartrate, and propylene glycol solutions. Therapeutic doses of antagonists of the present invention may be administered simultaneously with insulin either in the same composition or in separate compositions.

In addition, plaques in the brains of Alzheimer's patients are associated with neuronal hyperplasia. This hyperplasia leads to an overproduction of galanin at these sites. The galanin antagonists of the present invention are thus useful tools for studying the pathology of this disease.

Galanin may also be an important diagnostic marker for tumors, particularly pheochromocytomas. The human galanin DNA sequence is thus useful as a probe in DNA-based diagnostic tests, and recombinant galanin may be used to generate antibodies for immunoassay diagnostic tests.

The following examples are offered by way of illustration, not by way of limitation.

EXAMPLES

Example 1 - Cloning of Human Galanin DNAs

Pheochromocytoma specimens were obtained from multiple patients and pooled. The tissue was fragmented by mortar and pestle in liquid nitrogen and solubilized in extraction buffer (4 M guanidinium thiocyanate, 0.1 M Tris-HCl, pH 7.5, 1% β-mercaptoethanol, 0.5% sodium lauryl sarcosinate). The fragmented tissue was homogenized for 20 seconds using a tissue homogenizer. Phenol:chloroform:isoamyl alcohol (50:48:2) was added and the mixture was vortexed and centrifuged. Nucleic acids

were precipitated with isopropanol, and the pellet was resuspended in extraction buffer and precipitated again with isopropanol. The RNA pellet was sequentially washed with 75% and 100% ethanol. Poly $(A)^+$ RNA was purified using oligo d(T)-cellulose column chromatography as described by Sambrook et al., eds. (Molecular Cloning: A Laboratory Manual, vol. 1, 7.26-7.29, Cold Spring Harbor Laboratory Press, 1989).

First strand cDNA was synthesized from the poly $(A)^+$ RNA by first incubating 1.0 µg of poly$(A)^+$ RNA at 65°C for 3 minutes in 5 mM Tris-HCl pH 7.6, 0.05 mM EDTA. The RNA was cooled on ice, and the cDNA synthesis reaction was primed with 5 pmol of oligonucleotide ZC2487 (Table 1, Sequence ID Number 4) in a 10 µl reaction volume containing 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM each deoxynucleotide triphosphate, and 200 units of MMLV (RNase H^-) reverse transcriptase (GIBCO-BRL, Gaithersburg, MD). The reaction mixture was incubated at 45°C for 1 hour, following which the mixture was diluted with 180 µl of 10 mM Tris-HCl pH 7.6, 1 mM EDTA and stored at 4°C.

Table 1

Oligonucleotide Sequences (5′ to 3′)

ZC1159 (Sequence ID Number 3)

TTG TCC AAG CTT ACA CCT TC

ZC2487 (Sequence ID Number 4)

GAC TCG AGT CGA CAT CGA TCA GTT TTT TTT TTT TTT TTT

ZC2488 (Sequence ID Number 5)

GAC TCG AGT CGA CAT CGA TCA GCC CCC CCC CC

ZC2489 (Sequence ID Number 6)

GAC TCG AGT CGA CAT CGA TCA G

ZC3518 (Sequence ID Number 7)

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                A
TCT AGA ATT CAA GGA GAA GAG AGG CTG GAC
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ZC3520 (Sequence ID Number 8)

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                G   G   G
CCA TGG ATC CAG GGC CCC CGC CTC TTT
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ZC3757 (Sequence ID Number 9)

CCA TGG ATC CCA GAA ACT CAA TGA TTG TGC GCA T

Galanin cDNA sequences were amplified using degenerate primers encoding galanin DNA sequences using the polymerase chain reaction (PCR) method (Mullis et al., ibid.) Each of these primers also contained a 5′ tail of 10 nucleotides, which provided convenient restriction enzyme sites for subcloning. Three microliters of the cDNA solution and 50 pmol each of the oligonucleotide pools ZC3518 (Sequence ID Number 7) and ZC3520 (Sequence ID Number 8) (Table 1) were combined in a reaction volume of 50 μl containing 50 mM KCl, 10 mM Tris-HCl pH 9.0, 1.5

mM $MgCl_2$, 0.1% Triton X-100, 0.01% gelatin, 0.2 mM each deoxynucleotide triphosphate and 2.5 units of Thermus aquaticus (Taq) DNA polymerase (Promega Corporation, Madison, WI). The reaction mixture was overlaid with mineral oil and preheated to 94°C for five minutes. After 40 cycles (one minute at 94°C, two minutes at 48°C, three minutes at 72°C), the reaction was terminated with a 10 minute incubation at 72°C.

An aliquot of the reaction mixture was examined by electrophoresis on a 1.5% agarose gel, and a prominent fragment of approximately 250 bp in length was isolated. The fragment was digested with Eco RI and Bam HI and ligated to pUC19 plasmid DNA, which had been previously digested with Eco RI and Bam HI. The ligation mixture was precipitated in the presence of 2 μg high molecular weight oyster glycogen (Sigma Chemical Co., St. Louis, MO), resuspended in 3 μl of water, and electroporated into E. coli DH10B cells using 2.5 kV, 25 μFarads and 200 ohms. Inserts were detected by agarose gel analysis of PCR reactions performed on selected transfected cells using oligonucleotide pools ZC3518 (Sequence ID Number 7) and ZC3520 (Sequence ID Number 8) (Table 1). The reactions were carried out by inoculating a reaction mixture with the selected transformant under the conditions set forth above, and the samples were preheated for five minutes at 94°C. After 30 cycles (one minute at 94°C, one minute at 48°C, one minute at 72°C), aliquots of the reactions were electrophoresed in an agarose gel. The presence of an approximately 250 bp fragment indicated that the transformants contained an insert. Template DNA was prepared for double-stranded DNA sequencing and the DNA sequences of PCR products from 2 independent reactions were determined. The sequence of a representative galanin cDNA (clone 1-1) and the inferred amino acid sequence are shown in Figure 1 (Sequence ID Numbers 1 and 2). The first 3 amino acid codons of the mature human galanin sequence shown in Figure 1 (Sequence ID Number 1) were

derived from the PCR primer. Subsequent hybridization experiments suggest that these codons are present in the human sequence and that human galanin has the amino-terminal sequence gly-trp-thr-.

In order to extend the cloned sequence in the 5' direction, galanin-specific first strand cDNA was prepared as described above using 5 pmol of ZC3520 (Sequence ID Number 8) as the galanin-specific primer. The reaction was terminated by the addition of KOH to a concentration of 0.5 M and EDTA to a concentration of 50 mM followed by an incubation at 65°C for 5 minutes. After incubation, 1 ml of 50 mM KOH, 0.1 mM EDTA was added to the reaction, and the material was centrifuged through a Centricon 100 concentrator in a Sorvall SS-34 rotor at 6000 rpm for 5 minutes. One milliliter of 50 mM KOH, 0.1 mM EDTA was added to the spin column, and the column was centrifuged again. The flow-through was discarded, and the retentate was collected by centrifugation in the SS-34 rotor at 2000 rpm for 2 minutes. The solution was neutralized by the addition of 20 µl of 100 mM HCl and 10 µl 2 M Tris-HCl, pH 7.0. The cDNA was precipitated with ammonium acetate and ethanol in the presence of 5 µg of oyster glycogen. The cDNA was pelleted, washed with 75% ethanol, dried briefly and resuspended in 5.9 µl of water. The cDNA was d(G)-tailed by incubation at 35°C for 10 minutes with 0.1 M potassium cacodylate pH 7.2, 2 mM $CoCl_2$, 0.2 mM dithiothreitol, 0.1 mM dGTP and 19 units of terminal deoxynuceotidyl transferase (Pharmacia). The reaction was terminated by the addition of EDTA to 50 mM, and the cDNA was precipitated by ammonium acetate and ethanol in the presence of 2 µg of oyster glycogen. The cDNA was pelleted, washed with 75% ethanol, dried briefly and resuspended in 20 µl water. Second strand cDNA synthesis was performed in a 100 µl reaction volume containing 5 pmol ZC2488 (Table 1; Sequence ID Number 5), 50 mM NaCl, 10 mM Tris-HCl pH 8.3, 2 mM $MgCl_2$, 0.2 mM each deoxynucleotide triphosphate, and 0.01% gelatin. The

mixture was heated to 94°C for 3.5 minutes, then 4.5 units of Taq DNA Polymerase was added, and the mixture was overlaid with 50 μl of mineral oil. The temperature of the reaction mixture was dropped to 40°C for 5 minutes and then increased to 72°C for 15 minutes. The reaction was terminated by the addition of EDTA to a final concentration of 2.5 mM, and the reaction mixture was extracted with chloroform. The cDNA was precipitated with sodium acetate and ethanol in the presence of 5 μg oyster glycogen. The cDNA was pelleted, washed with 75% ethanol, dried, resuspended in 100 μl of water and stored at 4°C.

Complementary DNA encoding the 5′ sequences of galanin were amplified using 5′ rapid amplification of cDNA ends (RACE) (Frohman et al., Proc. Natl. Acad. Sci. USA 85: 8998-9002, 1988). After preheating the reaction mixture for five minutes at 94°C, 5 μl of the specific-primed, double-stranded cDNA template was amplified in a 50 μl reaction volume containing 50 pmol each of the oligonucleotides ZC2489 and ZC3757 (Table 1; Sequence ID Nos. 6 and 9) for 40 cycles (one minute at 94°C, two minutes at 65°C, three minutes at 72°C). The reaction was terminated with an incubation at 72°C for ten minutes. An aliquot of the reaction was analyzed by gel electrophoresis. The PCR fragments were isolated, digested with Sal I and Bam HI, then ligated to pUC19 plasmid DNA which was previously digested with Sal I and Bam HI. The ligation mixture was precipitated in the presence of 2 μg of oyster glycogen, and the DNA was resuspended in 3 μl of water, electroporated into E. coli DH10B cells and plated as described above. Selected transformants were examined for cDNA inserts by PCR analysis as described above using oligonucleotides ZC2489 and ZC3757 (Table 1; Sequence ID Nos. 6 and 9). Template DNA was prepared and the DNA sequences of PCR products were determined.

One μg of human genomic DNA is combined in a 50 μl volume reaction mixture as described above with 50 pmol each of the oligonucleotides ZC3518 and ZC3757 (Table 1; Sequence ID Nos. 7 and 9). After preheating the mixture to 95°C for five minutes, the DNA is amplified for 5 cycles of (one minute at 94°C, two minutes at 56°C, three minutes at 72°C), followed by 35 cycles of (one minute at 94°C, two minutes at 65°C, three minutes at 72°C). The reaction is terminated with a 10 minute incubation at 72°C. An aliquot of the reaction mixture is analyzed by agarose gel electrophoresis and the prominent band is isolated, digested with Eco RI and Bam HI, and ligated to pUC19 plasmid DNA previously digested with Eco RI and Bam HI. The ligation mixture is precipitated in the presence of 2 μg of oyster glycogen. The DNA pellet is resuspended in water, electroporated into E. coli DH10B cells and plated as described above. Selected transformants are picked and the presence of cDNA inserts is detected by PCR as described above using oligonucleotides ZC3518 and ZC3757 (Table 1; Sequence ID Nos. 7 and 9). Template DNA is prepared and the DNA sequences of PCR products are determined.

Example 2 - Construction of the Yeast Expression Vector pBS114

Plasmid pEAS102, comprising portions of the yeast vectors YIp5 and pJDB207, was constructed as follows. Plasmid pJDB207 (Beggs, Proceedings of Alfred Benzon Symposium 16: 383-389, "Molecular Genetics in Yeast," Copenhagen, Denmark, 1981), a derivative of pJDB219 (Beggs, ibid., 1978), was digested with Bam HI and Pst I to isolate the 4.4 kb fragment comprising the leu2-d gene, 2 micron plasmid and pBR322 sequences. Plasmid YIp5 (Struhl et al., ibid.) was subjected to partial digestion with Pst I and complete digestion with Bam HI to isolate the 4.3 kb fragment comprising the URA3 gene and pBR322

sequence. These two fragments were ligated and the resultant plasmid was designated pEAS102.

The Hind III sites in plasmid pEAS102 was destroyed by first digesting pEAS102 with Hind III to completion. The linearized plasmid was then incubated with the DNA polymerase I (Klenow fragment) in the presence of nucleotide triphosphates, recircularized by treatment with T4 DNA ligase and transformed into E. coli strain HB101. DNA prepared from the resulting transformants was screened for those plasmids which could no longer be linearized by digestion with Hind III.

To construct a yeast expression vector, the promoter and terminator regions from the Saccharomyces cerevisiae TPI1 gene along with the alpha factor prepro sequence were inserted into the pEAS102 derivative described above. The TPI1 promoter and alpha factor prepro sequence were obtained from plasmid pTGFαm. The plasmid pTGFαm was derived from plasmid pB12, which contained the TPI1 promoter, the MFα1 signal sequence, PDGF-BB sequence, the TPI1 terminator and pIC19R vector sequences. The construction of pB12 is disclosed by Murray et al. (U.S. Patent No. 4,766,073, which is incorporated herein by reference). The MFα1 signal sequence and PDGF-BB sequence were subcloned as an Eco RI-Xba I fragment into M13. The Sst I site present in the MFα1 signal sequence was changed to a Hind III site by in vitro mutagenesis using the method described by Kunkel et al. (U.S. Patent Number 4,873,192) and oligonucleotide ZC1159 (Table 1, Sequence ID No. 3). A clone having a Hind III site in place of the Sst I site was identified. A fragment containing the MFα1 signal sequence was isolated as an Eco RI-Hind III fragment.

The Eco RI-Hind III fragment containing the MFα1 signal sequence and a Hind III-Xba I fragment containing a synthesized transforming growth factor α (TGFα) coding sequence were ligated with Eco RI-Xba I linearized pUC13. The resultant plasmid, designated αfTGFα/pUC13, was

digested with Eco RI and Xba I to isolate the MFα1-TGFα insert, which was cloned into p170CB/pBR. The construction of plasmid p170CB/pBR is described by Murray (U.S. Patent Application Serial No. 07/557,219, which is incorporated herein by reference), and contains the TPI1 promoter, MFα1 signal sequence, PDGF-BB coding sequence, the TPI1 terminator and pBR322 vector sequences. Plasmid pB170CB/pBR was digested with Eco RI-Xba I to isolate the fragment containing the TPI1 promoter, pBR322 vector sequence and the TPI1 terminator. The Eco RI-Xba I pB170CB/pBR fragment and the Eco RI-Xba I MFα1-TGFα fragment were ligated. The resulting plasmid, designated TGFαCB, was digested with Cla I and Bam HI to isolate the expression unit, which was then subcloned into the yeast expression vector pMPOT2 (a yeast 2 micron-based plasmid containing yeast and bacterial replication origins, ampicillin resistance gene and POT1 selectable marker; deposited with American Type Culture Collection, Rockville, MD. under accession number 67788) to construct pTGFαm. Plasmid pTGFαm was digested with Bgl II and Hind III to isolate the 1236 base pair fragment containing the TPI1 promoter and MFα1 signal sequence.

The *Saccharomyces cerevisiae* TPI1 terminator fragment was obtained from plasmid pFG1 (Alber and Kawasaki, ibid.). It encompassed the region from the penultimate amino acid codon of the TPI1 gene to the Eco RI site approximately 700 base pairs downstream. A Bam HI site was substituted for the unique Eco RI site of pFG1 by first digesting the plasmid with Eco RI, then blunting the adhesive ends with DNA polymerase I (Klenow fragment), adding synthetic Bam HI linkers (CGGATCCA), and re-ligating to produce plasmid p136. The TPI1 terminator was then excised from p136 as an Xba I-Bam HI fragment. This fragment was ligated into YEp13 (Broach et al., ibid.), which had been linearized with Xba I and Bam HI. The resulting plasmid is known as p213. The Hind III site was then removed from the TPI1 terminator region of p213 by

digesting the plasmid with Hind III, blunting the resultant termini with DNA polymerase I (Klenow fragment), and recircularizing the linear molecule using T4 DNA ligase. The resulting plasmid was designated p270.

Alternatively, p270 may be constructed by digesting plasmid pM220 (deposited with American Type Culture Collection as an E. coli RR1 transformant, accession number 39853) with Xba I and Bam HI, purifying the TPI1 terminator fragment (approximately 700 bp) and inserting this fragment into Xba I-Bam HI digested YEp13.

The TPI1 terminator was removed from plasmid p270 as an Xba I-Bam HI fragment. This fragment was cloned into pUC19 along with another fragment containing the TPI1 promoter joined to the CAT (chloramphenicol acetyl transferase) gene to obtain a TPI1 terminator fragment with an Eco RV end. The resultant plasmid was designated pCAT. The TPI1 terminator was then removed from pCAT as an Eco RV-Bam HI fragment and cloned into pIC19H (Marsh et al., ibid.), which had been linearized with the same enzymes, to obtain plasmid pTTI. Plasmid pTTI was then digested with Hind III and Sal I to isolate the 718 bp TPI1 terminator fragment.

The 1236 base pair Bgl II-Hind III TPI1 promoter-MFα1 fragment and the 718 base pair Hind III-Sal I TPI1 terminator fragment were ligated with the pEAS102 derivative that have been linearized by digestion with Bam HI and Sal I. The ligation mixture was transformed into E. coli strain HB101, and plasmid DNA prepared from selected transformants was screened by restriction analysis to identify a clone bearing a plasmid of the correct structure. A positive clone was designated pBS114 (Figure 2).

Example 3 - Construction Of Yeast Strains Secreting Rat And Human Galanin

Plasmids useful in the secreted expression of galanin from yeast were constructed by inserting pairs of synthetic oligonucleotides (Sequence ID Numbers 10 and 11; Table 2) into pBS114. The oligonuceotides contain a galanin coding sequence flanked by bridge sequences which correctly link the galanin coding sequence to the expression and secretion elements within pBS114. The coding region for galanin was designed to utilize nucleotide triplets corresponding to codons which occur in highly expressed yeast genes. Since the same coding sequence will be used in mutagenesis (see Example 4) it was also useful to choose codons which provided the greatest variety of amino acid replacements from single base changes and create restriction endonuclease sites within the sequence. Where possible, codons which were a single base change away from chain termination codons were avoided.

Table 2

Oligonucleotide Sequences (5′ to 3′)

ZC3762 (Sequence ID Number 10)

5′ AGC TTA GAT AAG AGA GGT TGG ACC TTG AAC TCT GCA
GGT TAC TTG TTG GGT CCA CAC GCT ATC GAT AAC CAC
CGT TCT TTC TCT GAT AAG CAC GGT TTG ACC GGT TGA
ATT CA 3′

ZC3763 (Sequence ID Number 11)

5′ GAT CTG AAT TCA ACC GGT CAA ACC GTG CTT ATC AGA
GAA AGA ACG GTG GTT ATC GAT AGC GTG TGG ACC CAA
CAA GTA ACC TGC AGA GTT CAA GGT CCA ACC TCT CTT
ATC TA 3′

ZC3842 (Sequence ID Number 12)

5′ AGC TTA GAT AAG AGA GGT TGG ACC TTG AAC TCT GCA
GGT TAC TTG TTG GGT CCA CAC GCT GTT GGT TCT CAC

CGT TCT TTC TCT GAT AAG AAC GGT TTG ACC TCT TGA
ATT CA 3′

ZC3843 (Sequence ID Number 13)

5′ GAT CTG AAT TCA AGA GGT CAA ACC GTT CTT ATC AGA
GAA AGA ACG GTG AGA ACC AAC AGC GTG ACC CTT CTT
GAT ACC TGC AGA GTT CAA GGT ACC TCT CTT ATC TA
3′

The oligonucleotides were annealed to form 119 a 109 base pair duplex DNA with four-base 3′ overhangs at either end. These overhangs allowed the oligonucleotides to be annealed and ligated to pBS114 following digestion of the vector with Hind III and Bgl II restriction endonucleases. This placed the galanin coding sequence, which begins at nucleotide 16 of oligonucleotide ZC3762 (Sequence ID Number 10), after, and in the same reading frame as, the coding sequence for the secretion leader from yeast alpha mating factor. The first 15 bases of oligonucleotide ZC3762 (Sequence ID Number 10) include codons for the amino acid sequence LYS-ARG, which provides a site for the action of the product of the yeast KEX2 gene so that the alpha factor leader will be removed from galanin produced by yeast. The ligation mixture was transformed into E. coli, and plasmid DNA prepared from selected transformants was analyzed by restriction analysis. A plasmid having the correct rat galanin insert was chosen to transform into Saccharomyces cerevisiae.

The yeast-produced rat galanin is not amidated at its carboxy terminus, and is therefore a 30 amino acid polypeptide terminating in a glycine residue.

Using the criteria set forth for the preparation of oligonucleotides encoding rat galanin, oligonucleotides were synthezied for the construction of an expression vector containing human galanin. Oligonucleotides ZC3842 and ZC3843 (Table 2; Sequence ID Nos. 12 and 13), which were designed to encode human galanin using yeast-

preferred codons, were annealed to form a 109 base pair duplex DNA with four-base 3′ overhangs at either end.

The annealed oligonucleotides were ligated with pBS114 that had been linearized by digestion with Hind III and Bgl II, and the ligation mixtures were transformed into E. coli. The annealed oligonucleotides contained six mismatched base pairs which are noted as the underlined bases in ZC3843 (Sequence ID Number 13). As a result of the mismatches, plasmid DNA prepared from selected transformants was analyzed by restriction analysis and sequence analysis. A plasmid having the an insert having a sequence corresponding to ZC3842 (Sequence ID Number 12), the correct sequence for human galanin was chosen to transform into Saccharomyces cerevisiae.

Plasmids containing rat or human galanin coding sequences were transformed into Saccharomyces cerevisiae strain ZY100 (ade2-101 leu2-3 leu2-112 ura3-52 suc2-Δ9 gal2 pep4::TPI1p-CAT). Transformants were selected on -URADS plates (Table 3) and then restreaked for individual colonies on -LEUD plates (Table 3). These colonies were assayed for galanin secretion using the method described in Example 5.

Table 3

Media Recipes

-LeuThrTrp Amino Acid Mixture

4 g adenine
3 g L-arginine
5 g L-aspartic acid
2 g L-histidine free base
6 g L-isoleucine
4 g L-lysine-mono hydrochloride
2 g L-methionine
6 g L-phenylalanine
5 g L-serine
5 g L-tyrosine
4 g uracil
6 g L-valine

Mix all the ingredients and grind with a mortar and pestle until the mixture is finely ground.

-UraThrTrp Amino Acid Mixture

4 g adenine
3 g L-arginine
5 g L-aspartic acid
2 g L-histidine free base
6 g L-isoleucine
6 6 L-leucine
4 g L-lysine-mono hydrochloride
2 g L-methionine
6 g L-phenylalanine
5 g L-serine
5 g L-tyrosine
6 g L-valine

Mix all the ingredients and grind with a mortar and pestle until the mixture is finely ground.

-LEUD

20 g glucose

6.7 g Yeast Nitrogen Base without amino acids (DIFCO Laboratories Detroit, MI)

0.6 g -LeuThrTrp Amino Acid Mixture

18 g Agar

Mix all the ingredients in distilled water. Add distilled water to a final volume of 1 liter. Autoclave 15 minutes. After autoclaving add 150 mg L-threonine and 40 mg L-tryptophan. Pour plates and allow to solidify.

-LeuTrpThr Liquid Medium

20 g glucose

6.7 g Yeast Nitrogen Base without amino acids (DIFCO Laboratories Detroit, MI)

0.6 g -LeuThrTrp Amino Acid Mixture

Mix all the ingredients in distilled water. Add distilled water to a final volume of 1 liter. Autoclave 15 minutes. After autoclaving add 150 mg L-threonine and 40 mg L-tryptophan.

-URADS

20 g	glucose
6.7 g	Yeast Nitrogen Base without amino acids (DIFCO Laboratories Detroit, MI)
0.6 g	-UraThrTrp Amino Acid Mixture
182.2 g	sorbitol
18 g	Agar

Mix all the ingredients in distilled water. Add distilled water to a final volume of 1 liter. Autoclave 15 minutes. After autoclaving add 150 mg L-threonine and 40 mg L-tryptophan. Pour plates and allow to solidify.

Example 4 - Construction Of Galanin Analog Libraries Using Mutagenized Oligonucleotides

A library of coding sequences for galanin analogs is constructed using an adaptation of the method described by Hutchinson et al. (ibid.). Briefly, oligonucleotides were synthesized using phophoramidite solutions that have been purposely cross-contaminated such that the solutions that normally correspond to the four bases A, G, C, and T each contain small amounts of the other three phosphoramidites. After the four phosphoramidites have been dissolved at the concentration used for synthesis (0.13 M), three 60 µl aliquots were removed from each 10 ml supply bottle of the four phosphoramidite solutions. One aliquot from each phosphoramidite solution was then added back to the other three, resulting in four mixtures which were 98.2:0.6:0.6:0.6 in regards to the correct base and each of the other three. Using these mixtures in oligonucleotide synthesis will result in the production of pools of oligonucleotides that each contain one or more random base substitutions in the galanin coding sequence. Sets of oligonucleotide pools encoding rat and human galanin were prepared as described above. Each strand of each 113 base pair sequence was divided into three segments resulting in oligonucleotides of less than 42 bases in length. Because synthesis of each oligonucleotide pool begins with a 3′ base coupled to a support resin and as such cannot be conveniently mutagenized, breakpoints in the galanin sequences were chosen to result in 3′ ends that were in the third position of codons in which all four bases result in the same amino acid insertion. Mutagenesis was also limited to avoid certain codons, such as chain termination codons. Sites were also chosen to provide seven base cohesive 3′ overhanging ends after complementary pairs of oligonucleotides are annealed.

To mutagenize rat galanin, the oligonucleotide sequences shown in Table 4 (Sequence ID Nos. 14, 15, 16, 17, 18 and 19) were used as the starting sequence. The starting sequence of the generation of human galanin nalaogs were the oligonucleotide sequences shown in Table 4 (Sequence ID Nos. 14, 19, 21, 22 and 23). Uncontaminated phosphoramidites were used for the bases shown bold text of Table 4. Doped positions for the oligonucleotides shown in Table 4 are underlined.

Table 4

Mutagenic Oligonucleotide Pools (5′ to 3′)

ZC4153 (Sequence ID Number 14)
AGCTTAGATA AGAGAGGTTG GACCTTGAAC TCTGCAGGTT AC

ZC4154 (Sequence ID Number 15)
TTGTTGGGTC CACACGCTAT CGATAACCAC CGTTCTTTCT CT

ZC4301 (Sequence ID Number 16)
GATAAGCACG GTTTGACCGG TTGAATTCA

ZC4156 (Sequence ID Number 17)
GATCTGAATT CAACCGGTCA AACCGTGCTT ATCAGAGAAA

ZC4157 (Sequence ID Number 18)
GAACGGTGAT TATCGATAGC GTGTGGACCC AACAAGTAAC CT

ZC4158 (Sequence ID Number 19)
GCAGAGTTCA AGGTCCAACC TCTCTTATCT A

ZC4159 (Sequence ID Number 20)
TGTTGGGTCC ACACGCTGTT GGTTCTCACC GTTCTTTCTC T

ZC4160 (Sequence ID Number 21)
GAACGGTGAG AACCAACAGC GTGTGGACCC AACAAGTAAC CT

ZC4161 (Sequence ID Number 22)
GATAAGAAC GGTTTGACCT CTTGAATTCA

ZC4162 (Sequence ID Number 23)
GATCTGAATT CAAGAGGTCA AACCGTTCTT ATCAGAGAAA

Oligonucleotide pools ZC4153, ZC4154, and ZC4301 (Sequence ID Nos. 14, 15 and 16; Table 4) form the sense strand of the rat galanin insert; and ZC4156, ZC4157, and ZC4158 (Sequence ID Nos. 17, 18 and 19; Table 4) form the

antisense strand respectively in the 5′ to 3′ direction for each strand. Similarly ZC4153, ZC4159, ZC4161, ZC4162, ZC4160 and ZC4158 (Sequence ID Nos. 14, 20, 22, 23, 21 and 19, respectively; Table 4) form the sense and antisense strands for the human galanin library.

Oligonucleotide pools that contained 5′ ends that were internal to each 113 base pair insert sequence (i.e. ZC4154, ZC4301, ZC4157, ZC4159, ZC4161, ZC4160, and ZC4158; Sequence ID Nos. 15, 16, 18, 20, 22, 21 and 19, respectiively; Table 4) were treated with polynuleotide kinase in the presense of 1 mM ATP at 37°C for one hour to add the 5′ phosphate groups required for T4 DNA ligase activity. External 5′ ends were not treated in order to prevent tandem inserts from forming. After the kinase reaction was terminated by treatment at 85°C for ten minutes, equimolar amounts of the pools needed for each galanin insert were mixed in the two sets of six described above (ZC4153, ZC4154, ZC4301, ZC4156, ZC4157, and ZC4158 (Sequence ID Nos. 14, 15, 16, 17, 18 and 19, respectively; Table 4) for the rat galanin insert and ZC4153, ZC4159, ZC4161, ZC4162, ZC4160 and ZC4158 (Sequence ID Nos. 14, 20, 22, 23, 21 and 19, respectively; Table 4) for the human galanin insert). The mixtures were annealed by heating the mixtures to 85°C and allowing them to cool slowly to room temperature. The mixtures were ligated in the presence of ATP and T4 DNA ligase, and the ligation mixtures electrophoresed on a 1.2% agarose gel. The 113 base pair fragments were gel purified, and the fragments were each ligated with with pBS114 that had been linearized by digestion with Bgl II and Hind III. The ligation mixtures were transformed into E. coli strain DH10B by electroporation, and twelve individual transformant colonies were selected from each library for DNA sequence analysis. The remaining approximately 10,000 colonies in the rat library are pooled, as are approximately 10,000 human library clones, and both pools are used to prepare plasmid DNA for yeast transfomation.

Saccharomyces cerevisiae strain ZY100 (ade2-101 leu2-3 leu2-112 ura3-52 suc2-Δ9 gal2 pep4::TPI1p-CAT) are transformed with the pooled plasmid DNA from the DH10B transformants, and transformant colonies are selected on -URADS plates (Table 3). Individual URA^+ colonies are streaked on -LEUD plates (Table 3) and one colony from each streak is saved as a unique clone expressing one of the galanin library sequences.

Screening assays for galanin antagonists are performed on conditioned medium from cultures of individual transformant colonies derived from a library of yeast strains producing mutagenized galanin analogs. Conditioned medium is obtained by using each of the patched individual colonies from master plates to innoculate 200-300 microliters of liquid -LEUD medium (Table 3) in each well of 96-well microtiter plates and incubating the plates at 30°C for 36 to 48 hours. Clarified conditioned medium is obtained by centrifuging the microtiter plates for 5 minutes at 1000 X g and the medium is stored at 4°C for up to three days.

RIN5 cells are seeded into the wells of a 96-well microtiter plate at a density of 4 x 10^4 cells/well in 100 μl RPMI 1640 medium (GIBCO-BRL, Gaithersburg, MC) supplemented with 10% fetal bovine serum, 20 mM HEPES (Sigma, St. Louis, MO) 2 mM L-glutamine (GIBCO-BRL), 100 units/ml penicillin (GIBCO-BRL) and 100 μg/ml streptomycin (GIBCO-BRL) and grown at 37°C, 5% CO_2 atmosphere. Following one day of growth, the medium is removed from each well and replaced with 100 μl fresh medium. After an additional day of growth, the cells are washed three-times with 100 μl DMEM (GIBCO-BRL) supplemented with 2% BSA and 10 μg/ml aprotinin, leaving 50 μl of the medium in each well after the final wash.

Clarified conditioned from the yeast cultures is diluted ten-fold with RIN5 cell stimulation medium (DMEM (GIBCO-BRL, Gaithersburg, MD) supplemented with 2% BSA, 10 μg/ml aprotinin, 250 μM 7β-decaetyl-7β(γ-N-

methylpiperazino)-butyryl forskolin (Calbiochem, San Diego, CA), and 25nM rat galanin (Sigma, St. Louis, MO)). The RIN5 cells were stimulated by the addition of 50 µl of diluted conditioned medium. The following control wells are included with each assay:

A. Diluted conditioned medium from a Saccharomyces cerevisiae strain producing wild-type rat galanin.

B. Diluted conditioned medium from a Saccharomyces cerevisiae strain that does not produce galanin or galanin analog.

C. RIN cell stimulation medium without added conditioned medium.

D. RIN cell stimulation medium without galanin.

E. RIN cell stimulation medium without galanin or forskolin.

Thirty minutes after stimulation at 37°C, 20 µl is harvested from each well and diluted with 180 µl of insulin assay buffer (150 mM NaCl, 50 mM Na_2HPO_4, 25 mM EDTA, pH 7.4).

Each diluted sample is assayed for insulin content by radioimmunoassay. A 75 µl aliquot of each sample is transferred to individual wells of a 96-well LKB T-tray. Similarly, standard solutions containing known amounds of rat insulin (0, 0.25, 0.5, 1, 2, 5 and 10 ng/ml) are added, in duplicate, to control wells. To each well 75 µl of insulin assay buffer containing approximately 5 nCi of (3-[^{125}I]iodotyrosyl A14) human insulin (Amersham, Arlington Heights, IL) is added, followed by 75 µl of dilusted guinea pig anti-rat insulin serum (Linco Research, Inc., St. Louis, MO) and 75 µl of Staph protein-A coupled SPA reagent (Amersham). The T-tray is sealed and shaken overnight at room temperature. Counts bound to the SPA reagent are measured for each well by counding for 1 minute in an LKB beta plate reader. The counts are converted into insulin values by using the

standards to determine the approximate relationship between counts bound and insulin concentration. Galanin inhibits the forskolin-induced secretion of insulin and results in reduced insulin secretion. A galanin antagonist will reduce the galanin inhibition of insulin secretion in the assay system. Yeast strains making galanin antagonists result in more secreted insulin than yeast strains producing wild-type galanin, or galanin analogs without antagonist activity.

Yeast strains corresponding to the highest insulin values are selected for rescreening. The strains are grown and assayed in triplicate as described above. Galanin analogs that consistantly show higher levels of insulin secretion in the stimulation assay relative to a control strain making no analog are designated as galanin antagonists. Plasmid DNA recovered from strains producing galanin antagonists is subjected to DNA sequence analysis to determine the polypeptide sequence of potential galanin antagonists.

Example 5 - Synthesis of A Human Galanin Peptide

A human galanin peptide was synthesized on an Applied Biosystems model 431 Peptide Synthesizer (Foster City, CA) using the amino acid sequence predicted from the cDNA sequence. The product was purified by reversed-phase high performance liquid chromatography, and the identity of the peptide was confirmed by mass spectrometry and amino acid composition analysis.

An insulin secretion assay (Amiranoff et al., Eur. J. Pharm. 163: 205-207, 1989, which is incorporated by reference herein in its entirety), which relies on the galanin inhibition of insulin secretion from the cultured rat insulinoma cell line RIN5, was used to compare the bioactivity of the synthetic human galanin peptide with the bioactivities of rat galanin (Bachem, Inc, Torrance CA) and porcine galanin (Dr. Jean Rivier, Salk Institute,

La Jolla, CA). Briefly, triplicate assays were set up by culturing RIN5 cells in standard six-well tissue culture plates in RPMI 1640 medium (GIBCO-BRL) supplemented with 10% (v/v) heat-inactivated fetal calf serum (GIBCO-BRL), 24 mM $NaHCO_3$, 20 mM Hepes, 2 mM L-glutamine, 100 units/ml penicillin and 100 µl/ml streptomycin under conditions essentially described by Karlsen et al. (ibid.). Three days after plating, the medium was replaced with fresh medium. One or two days before the cells were assayed, the medium was removed and the cells in each well were washed with KRBB. After the second wash, the cells were incubated for two hours with 2 ml of KRBB. Following another wash KRBB, the cells were exposed to the galanin peptide by incubating the cells for one hour in 1 ml KRBB with amino acids (1x essential and non-essential minimun essential medium, GIBCO-BRL) in the presence of 5.6 mM glucose and in the presence of dilutions containing between 10^{-11} to 10^{-6} M of each species of galanin. After incubation, the medium was collected, and the amount of insulin released by the cells was measured using an insulin radioimunoassay essentially as described by Morgan and Lazarow (_Diabetes_ _12_: 115-126, 1963; which is incorporated by reference herein in its entirety) in which rat insulin was the standard. Total insulin secretion was reduced approximately 50% by human, porcine and rat peptides with the majority of the inhibitory effect on insulin secretion occuring at a concentration of 1 nM for all three peptides.

The effects of 100 mM saturating concentrations of each galanin peptide on basal and glucose-stimulated insulin secretion were examined by comparing insulin secretion using the assay described above in the presence of 0, 0.8 and 5.6 mM glucose. As shown in Table 5, the presence of 100 mM each galanin peptide in these assays did not inhibit basal insulin secretion, but markedly diminished the glucose-stimulated insulin release. Table 5, shows that insulin secretion by RIN5 cells was

stimulated approximately 3- and 6-fold by the presence of 0.8 and 5.6 mM glucose, respectively. The presence of human, porcine or rat galanin at 100 nM, a maximally effective concentration, produced equivalent inhibition of glucose-stimulated insulin release.

Table 5

Secreted Insulin Concentration (pM) in the Presence of 100mM galanin

Glucose (mM)	None	Human	Porcine	Rat
0	160 ■ 6.9	162 ■10	170 ■12	170 ■ 9.2
0.8	576 ■ 72	388 ■ 18	376 ■ 18	388 ■ 30
5.6	940 ■ 35	516 ■ 37	486 ■ 0	486 ■ 36

Example 6 - <u>In Vivo</u> Screening of Galanin Antagonists

A. Screening for Antagonists Capable of Reducing Galanin-Induced Inhibition of Insulin Secretion

Plasmid DNAs prepared from the yeast cells that are shown in Example 5 to secrete galanin antagonists are sequenced to determine the nucleotide sequence and deduced amino acid sequence of the galanin antagonists. Galanin antagonists are then synthesized from the deduced amino acid sequences on an Applied Biosystems Model 431A peptide synthesizer, and the peptides are purified by reverse-phase high-pressure liquid chromatography.

The synthetic galanin antagonists are tested for the ability to reduce galanin-induced inhibition of insulin secretion from the right (duodenal) lobe of a dog pancreas essentially as described by Dunning et al. (<u>Am. J. Physiol.</u> <u>251</u> (<u>Endocrinol. Metab.</u> 14): E127-E133, 1986, which is incorporated herein by reference in its entirety). Briefly, a synthetic galanin antagonist is infused for 30 minutes via the femoral vein at 2.5 or 25 pmol/kg/min in a pentobarbital-anesthetized, laparotomized dog (24-26 kg). At the same time, human or rat galanin is infused intravenously at doses needed to produce a 20%, 50% or 80% inhibition of insulin secretion (corresponding

to rates of 0.25 pmol/kg/min, 2.5 pmol/kg/min, or 25 pmol/kg/min, respectively). Blood is collected simultaneously from the femoral artery and the superior pancreaticoduodenal vein (SPVD) after surgical exclusion of venous drainage from the duodenum. Pancreatic venous blood flow is measured with an electromagnetic flow probe placed in an extracorporeal shunt from the SPVD to the portal vein. Alternately, the synthetic galanin may be infused directly into the pancreatic artery via a Clear-Cath® 22-G x 1 catherter (Abbott Hospital, Inc., North Chicago, IL).

Insulin secretion is measured using a radioimmunoassay essentially as described by Morgan and Lazarow (Diabetes 12: 115-126, 1963, which is incorporated herein in its entirety). Insulin secretion from the right (duodenal) lobe of the pancreas is calculated using the formula:

output = [venous (insulin) - arterial (insulin)] x pancreatic venous blood flow x (1 - hematocrit)

The results are compared with insulin level before the galanin infusion (basal level) and are expressed as percent of change from the basal level. The ability to prevent or reduce the 20%, 50%, and 80% galanin-induced inhibition of insulin secretion marks galanin antagonists that are active in the *in vivo* system.

Alternatively, the synthetic galanin antagonists are tested for their ability to reduce galanin-induced inhibition of insulin secretion in chronically catheterized rats essentially as described by Dunning and Taborsky (Diabetologia 33: 125-126, 1990, which is incorporated herein by reference in its entirety). Briefly, conscious, non-fasted rats bearing indwelling jugular cannulae are infused with a synthetic galanin antagonist at three doses, each one log unit apart, either in the presence or absence of galanin doses that would normally impair insulin secretion by 20%, 50% or 80%.

Insulin secretion is measured as described above. Galanin antagonists capable of preventing the galanin-induced inhibition or reduction of insulin secretion at the 20%, 50% and 80% levels are considered active antagonists in the *in vivo* assay.

B. Screening for Antagonists Capable of Preventing Galanin-Induced Inhibition of Insulin Secretion Before Nerve Stimulation

Galanin antagonists that are shown to be active against exogenous galainin will then be tested in three increasingly stringent assays for their ability to antagonize <u>endogenous</u> galanin action. First the antagonist will be given to 1) prevent circulating hepatic galanin from inhibiting insulin release when it is released during hepatic nerve stimulation, 2) to prevent locally released pancreatic galanin from impairing insulin release when given before splanchnic nerve stimulation, and 3) to prevent the decrease of basal insulin normally seen during mixed pancreatic nerve stimulation.

To assess whether a galanin antagonist is able to prevent circulating hepatic galanin from inhibiting insulin release when given before hepatic nerve stimulation, the galanin antagonist is administered to overnight-fasted adult mongrel dogs (25-30 kg) that are anesthetized using 30 mg/kg intravenous thiamylal sodium induction (Surital, Park-Davis, Morris Plains, NJ) followed by 1% halothane (Ayerst Laboratories, Inc., New York, NY) in 100% oxygen via mechanical ventilator. The femoral artery is cannulated for blood sampling and blood pressure measurement. The hepatic artery is catheterized with a Swan-Ganz catheter inserted into the femoral vein, passed up into the inferior vena cava and threaded into a main branch of a hepatic vein under radiographic guidance. A midline laparotomy is performed and a sampling catheter is placed in the portal vein within five centimeters of the porta hepatis. Ultrasonic probes (Transionic Inc.,

Ithica, NY) are placed around the hepatic artery and portal vein for measurement of blood flows into and out of the gut and liver.

Hepatic nerve stimulation is achieved by separating the neural sheath surrounding the hepatic artery from the vessel and placing the sheath in a bipolar electrode. To assure activation of only the sympathetic neural elements of the hepatic nerve and to eliminate reflex sympathetic input, a ganglionic blockade with hexamethonium bromide (Sigma Chemical, St. Louis, MO) and atropine is used. Intravenous boluses of 0.1 mg/kg of hexamethonium bromide are administered until the mean arterial pressure drops 10 mm Hg and remains at that level. Hexamethonium is then infused at 0.7 μg/kg/min x number of boluses required for the 10 mm Hg reduction of the mean arterial pressure. To eliminate parasympathetic influences on the liver, a 0.25 mg/kg atropine (Elkin-Sinn, Inc., Cherry Hill NJ) bolus is administered followed by an infusion of 0.4 μg/kg/min. The galanin antagonist is administered at 2.5 or 25 pmol/kg/min for fifteen minutes before and during the hepatic nerve stimulation. After administration of the antagonists, the hepatic nerve is stimulated for 10 minutes with square wave pulses of 8 Hz frequency, 10 mA current and 1 msec duration using a model S-44 Stimulator (Grass Instruments, Quincy, MA).

Blood samples are drawn simultaneously from the femoral artery, the portal vein and the hepatic vein before and during the antagonist infusion at five-15 minute intervals and aliqoted into tubes containing EDTA for anti-coagulation, and kept on ice until centrifuged. The plasma is then pipetted off and frozen for later analysis. Mean arterial pressure, hepatic arterial blood flow, portal vein blood flows and hematocrit are monitored throughout the experiments. Insulin levels are determined using the method described above.

To identify antagonists that are capable of preventing locally released pancreatic galanin from

impairing insulin release when given before splanchnic nerve stimulation, antagonists are administered to adult mongrel dogs using the method essentially described by Dunning et al. (Am. J. Physiol. 258 (Endocrinol. Metab. 21): E436-E444, 1990, which is incorporated herein by reference in its entirety). Briefly, overnight-fasted, adult mongrel dogs (19-37 kg) are anesthetized with 30 mg/kg intravenous thiamylal sodium (SURITAL, Parke Davis, Morris Plains, NJ). Anesthesia is maintained with 0.8% halothane (Ayerst Laboratories, Inc., New York, NY) administered from a calibrated vaporizer (Draeger, FRG) by mechanical ventilation in 100% oxygen. A laparotomy is performed on the anesthetized animal and an extracorporeal shunt containing a sampling port and an electromagnetic flow probe (Zepeda Instruments, Seattle, WA) is introduced between the superior pancreaticoduodenal vein and the portal vein. The femoral artery and vein are cannulated for arterial blood sampling and intravenous infusions of saline or the synthetic galanin antagonist.

Splanchnic nerves are dissected after bilateral thoracotomies are performed at the seventh intercostal space. The sympathetic trunks are dissected from the surrounding tissue along the dorsal rib cage, and bipolar electrodes (Harvard Apparatus, South Natick, MA) are placed on each nerve at the level of approximately T_{10}. The nerve trunks are then severed anterior to the electrodes. A sixty minute stabilization period follows the surgical procedures before experimentation.

The synthetic galanin antagonist is administered at 2.5 or 25 pmol/kg/min prior to the splanchnic nerve stimulation. The thoracic splanchnic nerves are stimulated by electrically stimulating the sympathetic trunks for ten minutes with square wave pulses of 1-ms duration and 10 mA current at a frequency of 8 Hz. The stimulation is performed with a model S-44 stimulator coupled to a PSIU6 stimulus isolation unit (Grass Instruments, Quincy, MA). Stimulation parameters are

monitored with an oscilloscope. Mean arterial blood pressure and superior pancreaticoduodenal vein blood flow is monitored continuously and hematocrit is determined at regular intervals. Paired blood samples are drawn from the femoral artery and the superior pancreaticoduodenal vein at five-15 minute intervals and aliqoted into tubes containing EDTA for anti-coagulation, and kept on ice until centrifuged. The plasma is then pipetted off and frozen for later analysis. Insulin levels are determined as described above. Insulin levels are calculated using the formula:

$$\text{output} = ([\text{insulin}]_{SPVD} - [\text{insulin}]_{FA}) \times (1 - \text{hematocrit}) \times \text{blood flow}_{SPVD}.$$

The inhibition of insulin secretion in the presence of antagonist is expressed as percentage change from basal baseline and is compared to that in the absence of antagonist. Significantly less inhibition of insulin release during the antagonist period is interpreted as successful reversal of the insulin inhibitory effect of neurally-released galanin.

Galanin antagonists capable of preventing the decrease of basal insulin normally seen during mixed pancreatic nerve stimulation are identified using the method essentially described by Dunning et al. (Am. J. Physiol. 256 (Endocrinol. Metab. 19): E191-E198, 1989; which is incorporated herein by reference in its entirety). Briefly, overnight-fasted adult male mongrel dogs are anesthetized as described above first with thiamylol and then with halothane. The femoral artery and vein are cannulated for blood sampling, blood pressure recording and continuous saline infusion. A midline laparotomy is performed to expose the duodenum and the associated lobe of the pancreas. An extracorporeal shunt containing a sampling port and an electromagnetic flow probe is then introduced between the superior pancreaticoduodenal vein and the portal vein. The

autonomic pancreatic nerves which course in the sheath of connective tissue surrounding the superior pancreaticoduodenal artery are isolated at their entrance into the pancreatic parenchyma and placed in a bipolar electrode (Harvard Apparatus, South Natick, MA). After a one hour stabilization period, baseline superior pancreaticoduodenal vein and femoral artery blood samples are obtained. The pancreatic nerve is stimulated by electrically stimulating the sympathetic trunks for ten minutes with square wave pulses of 1-ms duration and 10 mA current at a frequency of 8 Hz. The stimulation is performed with a model S-44 stimulator coupled to a PSIU6 stimulus isolation unit (Grass Instruments, Quincy, MA). Stimulation parameters are monitored with an oscilloscope. Blood sample are collected at five-15 minute intervals and aliqoted into tubes containing EDTA for anti-coagulation, and kept on ice until centrifuged. The plasma is then pipetted off and frozen for later analysis. The inhibition of insulin secretion in the presence of antagonist is expressed as percentage change from basal baseline and is compared to that in the absence of antagonist. Significantly less inhibition of insulin release during the antagonist period is interpreted as successful reversal of the insulin inhibitory effect of neurally-released galanin.

From the foregoing it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 07/662,221
(B) FILING DATE: 25-FEB-1991

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 242 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens
(F) TISSUE TYPE: Pheochromacytoma

(vii) IMMEDIATE SOURCE:
(B) CLONE: 1-1

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..240

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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GAA TTC AAG GAA AAG AGA GGC TGG ACC CTG AAC AGC GCG GGC TAC CTG       48
Glu Phe Lys Glu Lys Arg Gly Trp Thr Leu Asn Ser Ala Gly Tyr Leu
  1               5                  10                  15

CTG GGC CCA CAT GCC GTT GGC AAC CAC AGG TCA TTC AGC GAC AAG AAT       96
Leu Gly Pro His Ala Val Gly Asn His Arg Ser Phe Ser Asp Lys Asn
             20                  25                  30

GGC CTC ACC AGC AAG CGG GAG CTG CGG CCC GAA GAT GAC ATG AAA CCA      144
Gly Leu Thr Ser Lys Arg Glu Leu Arg Pro Glu Asp Asp Met Lys Pro
         35                  40                  45

GGA AGC TTT GAC AGG TCC ATA CCT GAA AAC AAT ATC ATG CGC ACA ATC      192
Gly Ser Phe Asp Arg Ser Ile Pro Glu Asn Asn Ile Met Arg Thr Ile
     50                  55                  60

ATT GAG TTT CTG TCT TTC TTG CAT CTC AAA GAC GCC GGC GCC CTG GAT      240
Ile Glu Phe Leu Ser Phe Leu His Leu Lys Asp Ala Gly Ala Leu Asp
 65                  70                  75                  80

CC                                                                   242
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 80 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Glu Phe Lys Glu Lys Arg Gly Trp Thr Leu Asn Ser Ala Gly Tyr Leu
 1               5                  10                  15

Leu Gly Pro His Ala Val Gly Asn His Arg Ser Phe Ser Asp Lys Asn
            20                  25                  30

Gly Leu Thr Ser Lys Arg Glu Leu Arg Pro Glu Asp Asp Met Lys Pro
        35                  40                  45

Gly Ser Phe Asp Arg Ser Ile Pro Glu Asn Asn Ile Met Arg Thr Ile
    50                  55                  60

Ile Glu Phe Leu Ser Phe Leu His Leu Lys Asp Ala Gly Ala Leu Asp
65                  70                  75                  80
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC1159

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TTGTCCAAGC TTACACCTTC 20

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC2487

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GACTCGAGTC GACATCGATC AGTTTTTTTT TTTTTTTTT 39

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC2488

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GACTCGAGTC GACATCGATC AGCCCCCCCC CC 32

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC2489

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GACTCGAGTC GACATCGATC AG 22

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC3518

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TCTAGAATTC AAGGARAAGA GAGGCTGGAC 30

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:
(B) CLONE: zc3520

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CCATGGATCC AGGGCCCGCT CTTT 24

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC3757

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CCATGGATCC CAGAAACTCA ATGATTGTGC GCAT 34

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 113 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:
(B) CLONE: 1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

AGCTTAGATA AGAGAGGTTG GACCTTGAAC TCTGCAGGTT ACTTGTTGGG TCCACACGCT 60

ATCGATAACC ACCGTTCTTT CTCTGATAAG CACGGTTTGA CCGGTTGAAT TCA 113

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 113 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC3763

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GATCTGAATT CAACCGGTCA AACCGTGCTT ATCAGAGAAA GAACGGTGGT TATCGATAGC 60

GTGTGGACCC AACAAGTAAC CTGCAGAGTT CAAGGTCCAA CCTCTCTTAT CTA 113

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 113 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC3842

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

AGCTTAGATA AGAGAGGTTG GACCTTGAAC TCTGCAGGTT ACTTGTTGGG TCCACACGCT 60

GTTGGTTCTC ACCGTTCTTT CTCTGATAAG AACGGTTTGA CCTCTTGAAT TCA 113

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 106 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC3843

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GATCTGAATT CAAGAGGTCA AACCGTTCTT ATCAGAGAAA GAACGTGAGA ACCAACAGCG 60

TGACCCTTCT TGATACCTGC AGAGTTCAAG GTACCTCTCT TATCTA 106

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC4253

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

AGCTTAGATA AGAGAGGTTG GACCTTGAAC TCTGCAGGTT AC 42

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC4154

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

TTGTTGGGTC CACACGCTAT CGATAACCAC CGTTCTTTCT CT 42

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC4301

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GATAAGCACG GTTTGACCGG TTGAATTCA 29

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC4156

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GATCTGAATT CAACCGGTCA AACCGTGCTT ATCAGAGAAA 40

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC4157

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GAACGGTGAT TATCGATAGC GTGTGGACCC AACAAGTAAC CT 42

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC4158

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GCAGAGTTCA AGGTCCAACC TCTCTTATCT A 31

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC4159

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

TGTTGGGTCC ACACGCTGTT GGTTCTCACC GTTCTTTCTC T 41

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC4160

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GAACGGTGAG AACCAACAGC GTGTGGACCC AACAAGTAAC CT 42

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC4161

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GATAAGAACG GTTTGACCTC TTGAATTCA 29

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC4162

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GATCTGAATT CAAGAGGTCA AACCGTTCTT ATCAGAGAAA 40

We claim:

1. A method for detecting the presence of a galanin antagonist comprising the steps of:

a) growing host cells containing a DNA construct capable of directing the expression of a galanin analog, the construct comprising the following operably linked elements: a transcriptional promoter, a DNA sequence encoding a galanin analog, and a transcriptional terminator, under growth conditions suitable for the expression of the galanin analog;

b) isolating the galanin analog encoded by the DNA sequence from the host cells;

c) exposing the isolated galanin analog in the presence of native galanin to a galanin receptor coupled to a response pathway under conditions and for a time sufficient to allow binding of the galanin analog to the receptor and an associated response through the pathway; and

d) detecting a reduction in the inhibition of the response pathway resulting from the binding of the galanin analog to the galanin receptor, relative to the inhibition of the response pathway by native galanin alone and therefrom determining the presence of a galanin antagonist.

2. A method according to claim 1 wherein the response pathway is a membrane-bound adenylate cyclase response pathway.

3. A method according to claim 2 wherein the step of detecting comprises measuring cyclic AMP production by the membrane-bound adenylate cyclase response pathway.

4. A method according to claim 1 wherein the galanin receptor is cell-associated and the step of detecting comprises measuring secretion of insulin or somatostatin.

5. A method according to claim 1 wherein the galanin receptor is membrane-bound in a cell-free extract or in a whole cell.

6. A method according to claim 1 wherein the galanin receptor is membrane-bound in an insulinoma cell membrane preparation.

7. A method according to claim 1 wherein the galanin receptor is membrane-bound in a whole cell, and the whole cell is an insulinoma cell.

8. A method according to claim 1 wherein the host cells are yeast host cells.

9. A method according to claim 1 wherein said DNA construct further comprises a secretory signal sequence operably linked to the DNA sequence encoding a galanin analog.

10. A method according to claim 1 comprising the additional steps of:

e) preparing said galanin antagonist in a substantially pure form;

f) exposing said galanin antagonist in the presence of native galanin to a galanin receptor coupled to a second response pathway under conditions and for a time sufficient to allow binding of the galanin analog to the receptor and an associated response through the pathway; and

g) confirming a reduction in the inhibition of the response pathway resulting from the binding of the galanin analog to the galanin receptor, relative to the inhibition of the second response pathway by native galanin alone and therefrom determining the presence of a galanin antagonist.

11. A method according to claim 10 wherein the second step of exposing (step (f)) comprises introducing the galanin analog into a whole animal.

12. A method according to claim 11 wherein the whole animal is a primate, a canine or a murine.

13. A method according to claim 10 wherein the second step of exposing comprises introducing the galanin analog into an isolated pancreas.

14. A method according to claim 13 wherein the pancreas is a canine pancreas or a murine pancreas.

15. A galanin antagonist produced from a host cell containing a DNA construct capable of directing the expression of a galanin antagonist, the construct comprising the following operably linked elements: a transcriptional promoter, a DNA sequence encoding a galanin antagonist, wherein the sequence encodes one or more amino acid residues that are different than the corresponding amino acid residues in native galanin, and a transcriptional terminator.

16. An isolated DNA molecule encoding a human galanin antagonist.

17. An isolated DNA molecule encoding human galanin.

18. A DNA molecule according to claim 17, wherein said molecule comprises the nucleotide sequence shown in Figure 1 (Sequence ID Number 1) from nucleotide number 28 to nucleotide number 108.

19. A DNA molecule according to claim 17, wherein said molecule comprises the nucleotide sequence shown in Figure 1 (Sequence ID Number 1) from nucleotide number 19 to nucleotide number 108.

20. An isolated human galanin.

21. An isolated recombinant human galanin produced from a host cell containing a DNA construct capable of

directing the expression of human galanin, the construct comprising the following operably linked elements: a transcriptional promoter, a DNA sequence encoding human galanin, and a transcriptional terminator.

FIGURE 1

```
GAA TTC AAG GAA AAG AGA GGC TGG ACC CTG AAC AGC GCG GGC TAC CTG     48
Glu Phe Lys Glu Lys Arg Gly Trp Thr Leu Asn Ser Ala Gly Tyr Leu
 1               5                  10                  15

CTG GGC CCA CAT GCC GTT GGC AAC CAC AGG TCA TTC AGC GAC AAG AAT     96
Leu Gly Pro His Ala Val Gly Asn His Arg Ser Phe Ser Asp Lys Asn
            20                  25                  30

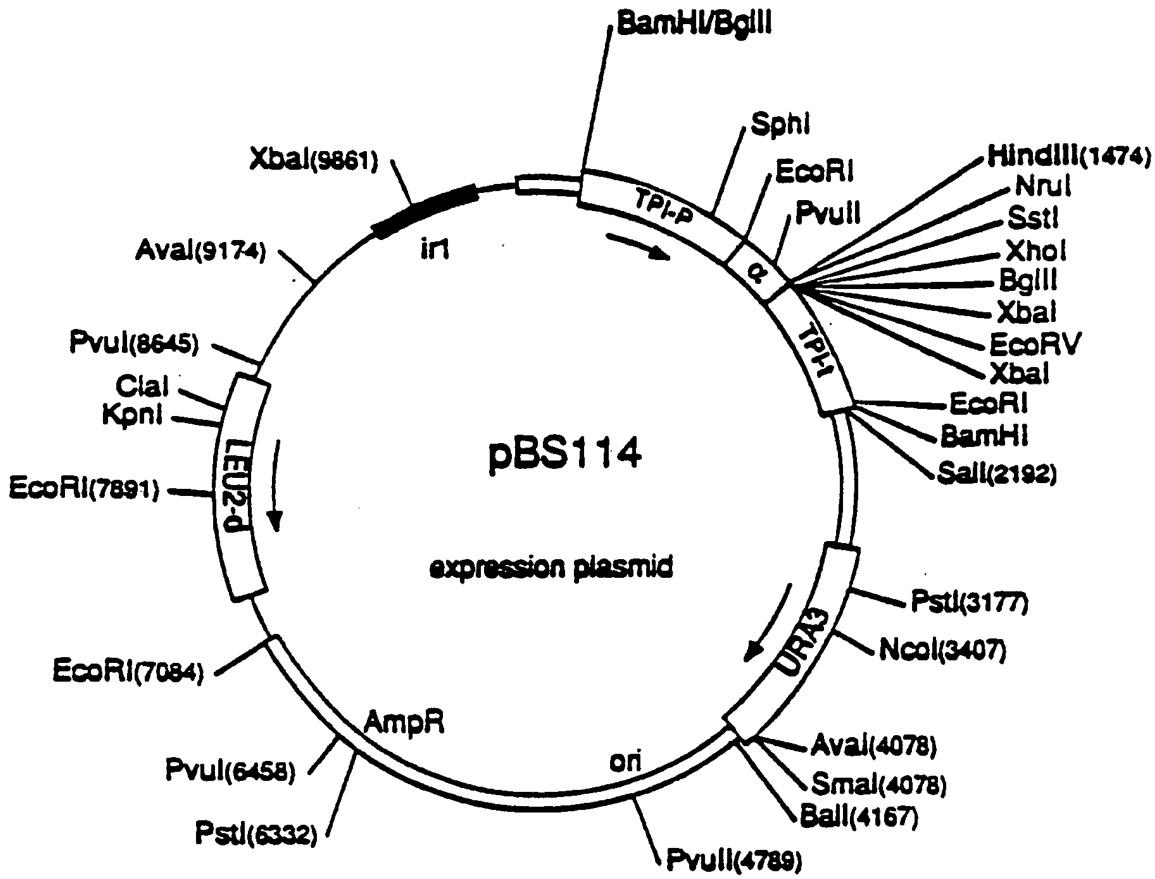
GGC CTC ACC AGC AAG CGG GAG CTG CGG CCC GAA GAT GAC ATG AAA CCA    144
Gly Leu Thr Ser Lys Arg Glu Leu Arg Pro Glu Asp Asp Met Lys Pro
        35                  40                  45

GGA AGC TTT GAC AGG TCC ATA CCT GAA AAC AAT ATC ATG CGC ACA ATC    192
Gly Ser Phe Asp Arg Ser Ile Pro Glu Asn Asn Ile Met Arg Thr Ile
    50                  55                  60

ATT GAG TTT CTG TCT TTC TTG CAT CTC AAA GAC GCC GGC GCC CTG GAT    240
Ile Glu Phe Leu Ser Phe Leu His Leu Lys Asp Ala Gly Ala Leu Asp
65                  70                  75                  80

CC                                                                 242
```

Figure 2


BamHI/BglII
SphI
EcoRI
PvuII
HindIII(1474)
NruI
SstI
XhoI
BglII
XbaI
EcoRV
XbaI
EcoRI
BamHI
SalI(2192)
TPI-P
α
TPI-t
XbaI(9861)
AvaI(9174)
ir1
PvuI(8645)
ClaI
KpnI
EcoRI(7891)
LEU2-d
pBS114
expression plasmid
URA3
PstI(3177)
NcoI(3407)
EcoRI(7084)
AmpR
ori
PvuI(6458)
PstI(6332)
AvaI(4078)
SmaI(4078)
BalI(4167)
PvuII(4789)

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 92/01469

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.5 G 01 N 33/68 G 01 N 33/567 C 12 N 15/16
C 07 K 7/10

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl.5	G 01 N C 12 N C 07 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
Y	WO,A,8906135 (AMYLIN CORP.) 13 July 1989, see page 29, lines 7-20; claim 49 ---	1,4,6,7,10
Y	Proceedings of the National Academy of Sciences of USA, vol. 83, September 1986, National Academy of Science, (Washington, DC, US), A. RÖKAEUS et al.: "Construction of a porcine adrenal medullary cDNA library and nucleotide sequence analysis of two clones encoding a galanin precursor", pages 6287-6291, see figures 1,2 ---	1,4,6,7,10,17-21
Y	Gastroenterology, vol. 91, 1986, Elsevier, (New York, US), F.E. BAUER et al.: "Distribution and molecular heterogeneity of galanin in human, pig, guinea pig, and rat gastrointestinal tracts", pages 877-883, see figure 3; tables 3,5 --- -/-	17-21

° Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
19-06-1992	03. 08. 92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	NURIA TORIBIO

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,X	FEBS Letters, vol. 283, no. 2, 3 June 1991, Elsevier Publishers, (Amsterdam, NL), M. BERSANI et al.: "Human galanin: primary structure and identification of two molecular forms", pages 189-194 ---	17-20
P,X	Proceedings of the National Academy of Sciences of USA, vol. 88, December 1991, National Academy of Sciences, (Washington, DC, US), W.E. SCHMIDT et al.: "Isolation and primary structure of pituitary human galanin, a 30-residue nonamidated neuropeptide", pages 11435-11439 ---	20
P,X	Diabetes, vol. 41, no. 1, January 1992, Am. Diabetes Assoc., (New York, US), G.L. McKNIGHT et al.: "Sequence of human galanin and its inhibition of glucose-stimulated insulin secretion from RIN cells", pages 82-87, see figures 1-5 ---	17-21
P,X	Endocrinology, vol. 129, no. 3, September 1991, The Endocrine Soc., (Baltimore, US), H.F. EVANS et al.: "Human galanin: Molecular cloning reveals a unique structure", pages 1682-1684, see figures 1,2 ---	17-19, 21
P,X	Supplement to Gastroenterology, vol. 100, no. 5, May 1991, Elsevier, (New York, US), R.M. STRAUSS et al.: "Molecular analysis of human galanin variant peptide structure and species-specific regulation of gene expression", & Digestive Disease Week and the 92nd Annual Meeting of the American Gastroenterological Association, New Orleans, Louisiana, 19-22 May 1991, see abstract -----	17-19

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9201469

SA 58200

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 17/07/92
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A- 8906135	13-07-89	AU-A- 2949489	01-08-89
		EP-A- 0348490	03-01-90
		JP-T- 3501611	11-04-91

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